Trace-level determination of flavonoids and their conjugates

de Rijke, E.

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Application to plants of the Leguminosae family

VRIJE UNIVERSITEIT

Trace-level determination of flavonoids and their conjugates
Application to plants of the Leguminosae family

ACADEMISCH PROEFSCHRIFT

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de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Exacte Wetenschappen
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in de aula van de universiteit,
De Boelelaan 1105

door

Eva de Rijke

geboren te Purmerend

promotoren:	prof.dr. C. Gooijer
	prof.dr. U.A.Th. Brinkman
copromotor:	dr. F. Ariese

Aan mijn ouders

Contents

1 Analytical separation and detection methods for flavonoids: an overview [1]

Summary

Flavonoids receive considerable attention in the literature, specifically because of their biological and physiological importance. This review focuses on separation and detection methods for flavonoids and their application to plants, food, drinks and biological fluids. The topics that will be discussed are sample treatment, column liquid chromatography (LC), but also methods such as gas chromatography (GC), capillary electrophoresis (CE) and thin-layer chromatography (TLC), various detection methods and structural characterization. Because of the increasing interest in structure elucidation of flavonoids, special attention will be devoted to the use of tandem-mass spectrometric (MS) techniques for the characterization of several important sub-classes, and to the potential of combined diode-array UV (DAD UV), tandem-MS and nuclear magnetic resonance (NMR) detection for unambiguous identification. Emphasis will be on recent developments and trends.

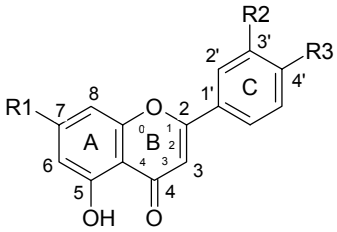
[1] E. de Rijke, Pieter Out, Wilfried M. A. Niessen, F. Ariese, U. A.Th. Brinkman, C. Gooijer, submitted for publication in J. Chromatogr. A.

Contents Chapter 1

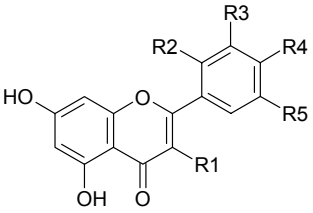
1.1 Introduction

Flavonoids are a large group of structurally related compounds with a chromane-type skeleton, with a phenyl substituent in the C_2 or C_3 position. The main flavonoid subclasses are depicted in Fig. 1. Flavonoids are often hydroxylated in positions 3, 5, 7, 3', 4' and/or 5'. Frequently, one or more of these hydroxyl groups are methylated, acetylated, prenylated or sulphated. In plants, flavonoids are often present as *O*- or *C*-glycosides. The *O*-glycosides have sugar substituents bound to a hydroxyl group of the aglycone, usually located at position 3 or 7, whereas the *C*-glycosides have sugar groups bound to a carbon of the aglycone, usually 6-C or 8-C. The most common carbohydrates are rhamnose, glucose, galactose and arabinose. Flavonoid diglycosides are also frequently found. Two very common disaccharides contain glucose and rhamnose, 1→6 linked in neohesperidose and 1→2 linked in rutinose. The sugars are often further substituted by acyl residues such as malonate and acetate [1]. Flavonoids are referred to as glycosides when they contain one or more sugar groups (or glucosides in case of a glucose moiety), and as aglycones when no sugar group is present.

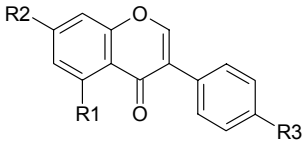
Given the above structural variety, it will come as no surprise that there is an extremely large number of flavonoids. Typical quotations include " > 4000 known flavonoids comprising 12 subclasses" [2], "More than 3000 flavones and more than 700 known isoflavones exist in plants" [3] and "Almost 6500 different flavonoids are known" [4]. Consequently, the separation, identification and trace-level determination of flavonoids is challenging. They receive considerable attention in the literature, specifically because flavonoids are of biological and physiological importance. Flavonoids are one of the largest groups of secondary metabolites, and they play an important role in plants as defence and signalling compounds in reproduction, pathogenesis and symbiosis [5, 6]. Plant flavonoids are involved in response mechanisms against stress, as caused by elevated UV-B radiation [7–10], infection by microorganisms [11] or herbivore attack [12]. Flavonoids are also involved in the production of root nodules as a nitrogen fixation system after infection by *Rhizobium* bacteria in a variety of leguminous plants [13] – they are pigment sources for flower colouring compounds – [14] and play an important role in interactions with insects [15]. They also affect human and animal health because of their role in the diet, which is ascribed to their antioxidant properties [16] or their estrogenic action [17], and to a wide range of antimicrobial and pharmacological activities [18, 19]. Many different enzymes involved in intracellular signalling can be affected by flavonoids. Especially the effects of flavonoids on protein kinases are of great interest since they directly influence immune functions in the host [20]. The above described spectrum of functions explains why recently quite a number of reviews have been published on the properties of flavonoids [8, 21–24] and on the state-of-the-art analysis of flavonoids (Table 1).



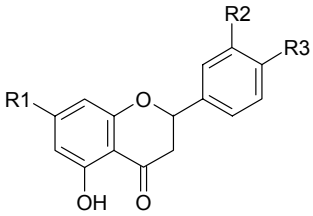
Flavone



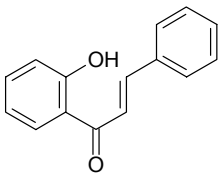
Flavonol



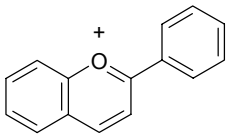
Isoflavone



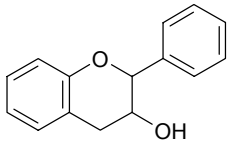
Flavanone



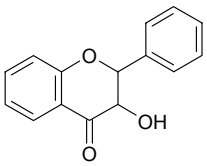
Chalcone



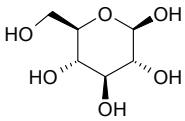
Anthocyanidin



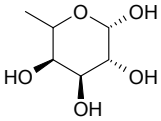
Catechin



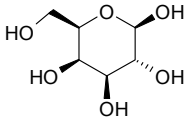
Flavanolol



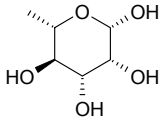
β-D-glucose



L-arabinose



β-D-galactose



L-rhamnose

Flavanone	R_1	R_2	R_3	MW
Hesperetin	OH	OH	OCH_3	302
Hesperidin	7-*O*-Glurha	OH	OCH_3	610
Naringin	7-*O*-Glurha	H	OH	580
Naringenin	OH	H	OH	272
Eriocitrin	7-*O*-Rut	OH	OH	596
Eriodyctiol	OH	OH	OH	288
Isosakurametin	OH	H	OCH_3	286

Flavone	R_1	R_2	R_3	R_4	MW
Chrysoeriol	OH	H	OCH_3	OH	300
Chrysin	OH	H	H	H	254
Apigenin	OH	H	H	OH	270
Luteolin	OH	H	OH	OH	286
Acacetin	OH	H	H	OCH_3	284
Genkwanin	H	OCH_3	H	OH	284

Flavonol	R_1	R_2	R_3	R_4	R_5	MW
Rutin	3-*O*-Glurha	H	OH	OH	H	610
Kaempferol	OH	H	H	OH	H	286
Quercetin	OH	H	OH	OH	H	302
Morin	OH	OH	H	OH	H	302
Isorhamnetin	OH	H	OCH_3	OH	H	316
Myricetin	OH	H	OH	OH	OH	318
Fisetin	OH	H	OH	OH	H	286

Isoflavone	R_1	R_2	R_3	MW
Biochanin A	OH	OH	OCH_3	284
Sissotrin	OH	7-*O*-Glu	OCH_3	446
Genistein	OH	OH	OH	270
Genistin	OH	7-*O*-Glu	OH	432
Formononetin	H	OH	OCH_3	268
Ononin	H	7-*O*-Glu	OCH_3	430
Daidzein	H	OH	OH	254
Daidzin	H	7-*O*-Glu	OH	416

Glu=Glucoside, Glurha=Glucorhamnosyl, Rut=Rutinose

Fig. 1. Structures and molecular weights of the main flavonoid subclasses, their principal sugar substituents, and selected flavonoids discussed in the review. The bond numbering of the C-ring and the position numbering of the carbon atoms are shown in the flavone structure.

An important aspect of flavonoid analysis is whether to determine the target analytes in their various conjugated forms or as the aglycones. In biological fluids (serum, plasma and urine) flavonoids exist as glucuronide and sulphate conjugates. In most cases, only the total aglycone content is determined; therefore, a hydrolysis step is used. However, in plants, medicine and food products, researchers are usually interested in the intact conjugates. For example, for the classification of plant species, intact flavonoid profiles in plants are determined [25–27]. In that case, analyses become much more complicated, because the number of target analytes increases significantly: much more selective and sensitive analytical methods are now required. In Fig. 2 the principal strategies for the determination of flavonoids in biological fluids, drinks, plants and food – the main sample types – are schematically depicted. The various steps in this flow chart will be considered in some detail below, with attention to both routine procedures and recent developments. Of course, in view of the complexity of the problem (almost) all analytical methods dealing with flavonoids include a high-performance separation method. The choice of the method depends on the sensitivity required for the purpose at hand, the complexity of the biological matrix – which is related to the time spent on sample pretreatment prior to analysis – the required chromatographic resolution and the preferred detection method.

Table 1. Recent reviews on the analysis of flavonoids.

Target compounds	Method	Matrix	Ref.
Sample handling			
Phenolic compounds		fruit	160
Phenolic compounds		food and plants	31
Bioactive phenols		food and plants	30
Instrumental techniques			
Naturally occurring substances	LC–MS	food	80
Flavonoids	LC–UV	food	20
Flavonoid glycosides	MS	all	79
Flavonoids	MS	all	148
Flavonoids	MS	biological samples	83
Phytochemicals	hyphenated techniques	legumes	161
Bioactive phenols	all	plants, fruit and vegetables	30
Oxidation products and antioxidants	LC–MS	biological systems	82
Phytoestrogens	all	all	162
Polyphenol phytoestrogens	all	food and body fluids	32
Phytochemicals	LC–DAD UV–MS	plants	81
Phytochemicals	CE	plants and food	120
Naturally occurring antioxidant phytochemicals	all	plants	100
Flavonoids	all	natural matrices	163

To give a general indication of the attention devoted to flavonoid analysis in the last five years, over fifty papers were written on the analysis of plants, mainly to characterize and quantify their constituents for medicinal or taxonomical reasons. Most reviews listed in Table 1 also deal with the determination of flavonoids in plants. A further fifty papers reported on the analysis of human and animal body fluids. The main goal of these studies was to monitor flavonoid metabolism. Some thirty papers and several reviews (*cf.* Table 1) were devoted to the analysis of flavonoids in food and drinks, in most cases to determine their anti-oxidant activity and, in the case of juices, also to check them for possible adulterants.

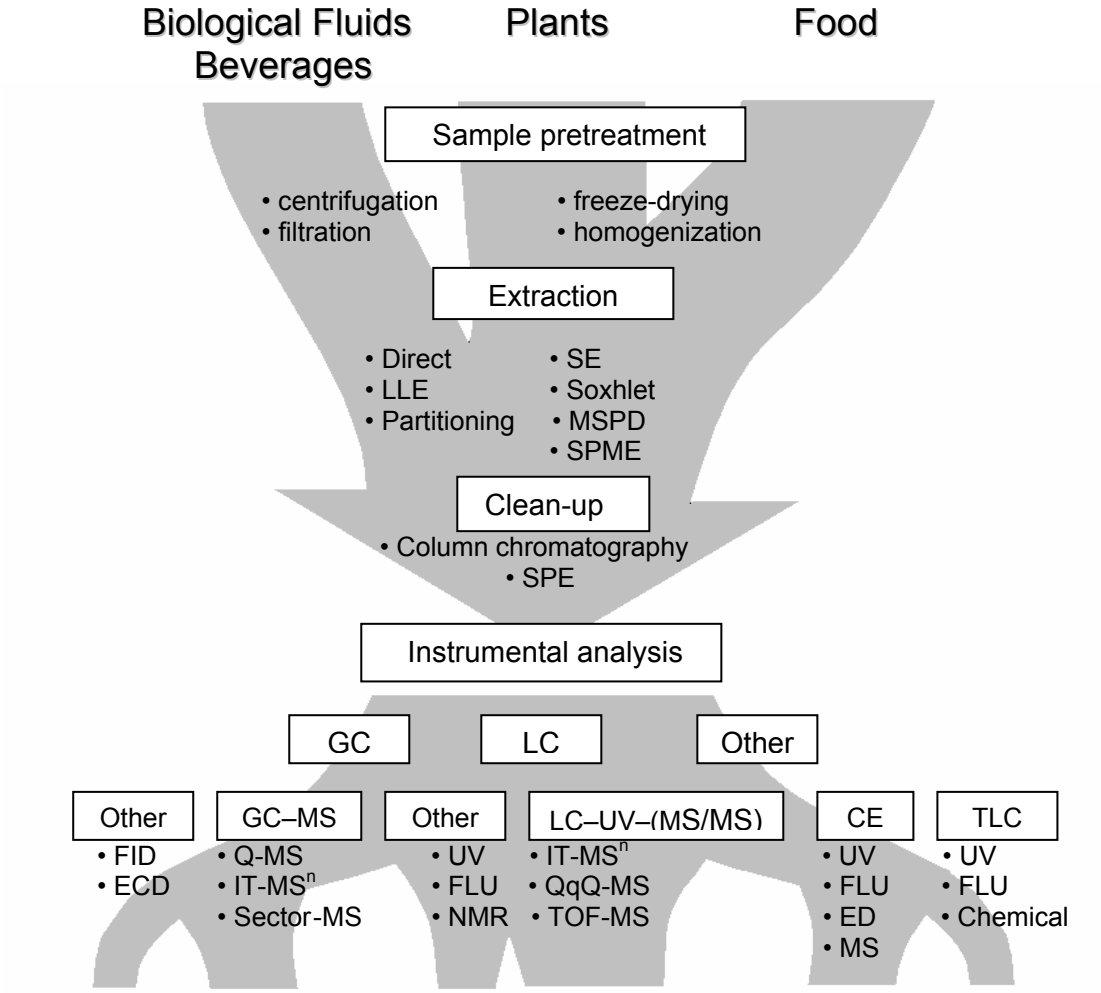


Fig. 2. Strategies for the determination of flavonoids in biological fluids, beverages, plants and food.

As was already briefly indicated above, the present review intends to discuss the determination of a wide variety of flavonoids – aglycones as well as conjugates – in many different sample types by means of routine or more recently developed analytical techniques. In all instances, selected real-life applications will be included to illustrate the practicability, and the scope and limitations of the various approaches. Because of the increasing interest in structure elucidation of flavonoids, special attention will be devoted to the use of tandem-mass

spectrometric (MS/MS) techniques for the characterization of several important sub-classes, and to the potential of combined diode-array UV (DAD UV), tandem-MS and nuclear magnetic resonance (NMR) detection for unambiguous identification. The structures of the main flavonoids discussed in the following sections are listed in Fig. 1.

1.2 Sample treatment

1.2.1 Analyte isolation

Over the years many sample pre-treatment methods have been developed to determine flavonoids in various sample types. There are three main types of flavonoid-containing matrices: plants, food and liquid samples such as biological fluids and drinks (*cf.* Fig. 2). The solid samples are usually first homogenized, which may be preceded by (freeze-)drying. The next step is analyte isolation. For this purpose, solvent extraction (SE) – which may be followed by solid-phase extraction (SPE) – is still the most widely used technique, mainly because of its ease of use and wide-ranging applicability. Soxhlet extraction is used less frequently to isolate flavonoids from solid samples. Liquid samples are usually first filtered and/or centrifuged, after which the sample is either directly injected into the separation system or, more often, the analytes are first isolated using liquid–liquid extraction (LLE) or SPE. Table 2 gives some recent representative examples of these procedures for flavonoid isolation. As regards SE and Soxhlet, in most cases aqueous methanol or acetonitrile is used as solvent. In the case of LLE the extraction solvent usually is ethyl acetate or diethyl ether containing a small amount of acid. LLE is usually directed at the isolation of aglycones, while the other methods can have the isolation of both aglycones and conjugates as their goal. If aglycones are the target analytes, chemical hydrolysis is usually performed – with hydrochloric acid or formic acid at elevated temperatures (80–100°C) or by refluxing with acid in the presence of ethanol – but enzymatic hydrolysis with β-glucuronidase or β-glucosidase is also used [28, 29]. If the interest is in the intact flavonoid glycosides, hydrolysis should of course be prevented. For more detailed information, the reader is referred to papers by Robards and co-workers who recently reviewed various sample preparation procedures for flavonoids [30, 31]. In other reviews these procedures received attention in the context of particular application areas, *e.g.* soy food and human biological fluids [32] and fruits [33].

Table 2. Representative examples of LLE, SE and Soxhlet extraction procedures for flavonoids.

Analytes	Solvent	Sample	Details	Analysis	Ref.
Soxhlet					
Various flavonoids	MeOH	*M. spicata, T. europea, U. dioica, H. perforatum*	extracted 12 h with methanol, evaporated, redissolved in phosphate buffer–methanol 80:20 (*v/v*).	GC–MS	104
Daidzein, genistein	MeOH–H_2O (9:1, v/v)	soybean milk, farina, meat	1 h temperature programme up to 130°C	LC–ED	77
Flavonoid glycosides	EtOH–H_2O (7:3, v/v)	*Ginkgo biloba* leaves		LC–UV	21
LLE					
(-)-Epicatechin gallate, epigalocatechin gallate	EtOAc–H_2O (1:1, v/v)	green tea	for analysis, EtAc–H_2O was used as the binary system	HSCCC	111
Epicatechin	Et_2O 0.1 M HCl (pH=2)	olive oil		LC–UV and FLU	70
Scrutellarein	EtOAc, 3% 1 M phosphoric acid	rat plasma		LC–UV	29
Quercitrin	Et_2OM 0.1 HCl 0.2 (pH=2)	red wine	dissolving dried extract in methanol–H_2O (1:1, *v/v*) improved separation efficiency	LC–UV	164
SE					
Epicatechin, catechin, rutin, apigenin, luteolin, quercetin	MeOH	*Ginkgo biloba* leaves	dried leaves sonicated with 5 mL methanol for 30 min	CE–ED	165
Isoflavone and flavonol glucoside-(di)malonates	MeOH–H_2O (9:1, v/v)	*T. pratense, T. dubium. T. repens, L corniculatus* leaves	dried leaves ground with methanol–H_2O, filtered and once more extracted; extracts combined	LC–UV–MS and FLU	98
Daidzin, glycitin, genistin, daidzein, glycitein, genistein	MeCN–H_2O (1:1, v/v)	soy food	hydrolysis avoided to determine malonates and acetates	LC–MS	166
Catechin, epicatechin, procyanidin, flavonols, anthocyanins, dihydrochalcones	Me_2O–H_2O (7:3, w/w).	apple		LC–UV and LC–MS	167

Abbreviations: MeCN: acetonitrile, EtOAc: ethyl acetate, Et_2O: diethyl ether, MeOH: methanol, EtOH: ethanol, Me_2O: acetone.

Sample treatment by means of SPE, matrix solid-phase dispersion (MSPD) and solid-phase micro-extraction (SPME) deserves some more attention. Although SPE is not a very new technique, it has only recently been applied in flavonoid analysis. Moreover, compared with traditional extraction methods, the techniques mentioned above can be easily automated, while solvent consumption is lower and analysis times are shorter.

1.2.2 Solid-phase extraction

Non-selective SPE on, typically, alkyl-bonded silica or copolymer sorbents is widely used for analyte extraction and enrichment from aqueous samples and sample extracts – primarily in

environmental, pharmaceutical and biomedical analysis. Its use in flavonoid analysis is, however, relatively new. In most cases the sorbent is C18-bonded silica and the sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids, which would reduce their retention. A recent example is the purification of methanolic extracts of olives [34]. After SE of the homogenized olives, the extract was evaporated to dryness, redissolved in water containing hydrochloric acid (pH 2) and loaded on a C18 sorbent. After washing with hexane to remove lipids, the flavonoids were eluted with pure methanol. Combining this procedure with LC–ESI(+)-MS resulted in the identification of up to eleven phenolic compounds in 29 types of olives. These included several flavonoid-(di)glycosides.

Another recent application is the determination of daidzein and genistein in plasma using LC–ESI(–)-MS/MS [35]. 250 µl of plasma were diluted and acidified with 0.5% formic acid before application to a C18 sorbent. Dilution and acidification were required to obtain satisfactory recoveries (*ca.* 80%), probably due to reduced flavonoid–protein interaction. After elution with methanol, off-line combination with the LC procedure gave LODs of 3 and 9 ng/ml for genistein and daidzein, respectively, when using multiple reaction monitoring (MRM).

In a less traditional application, SPE was used on-line after an LC separation but prior to MS and NMR detection, to effect enrichment of the analytes of interest in an oregano sample extract [36]. The use of expensive deuterated solvents during the LC separation could now be avoided – for LC a traditional acetonitrile–aqueous ammonium formate buffer gradient was used – but no solvent suppression was required since the flavonoids were eluted from the C18 sorbent with (a limited volume of) deuterated acetonitrile. A multiple trapping process was used to further concentrate the analytes and thereby reduce the acquisition time of the off-line NMR measurements. With this LC–monitoring UV–SPE/NMR–MS method five flavonoids, a phenolic acid and a monoterpene were identified in the oregano sample. The LC–NMR method will be further discussed in Section 4.3.

For the analysis of a red clover extract a dual-SPE method was used [3]. The methanolic extract was subjected to SPE and fractions were collected and transferred to a second SPE sorbent. Three sorbents were tested and the pH and organic mole fraction of the aqueous organic solvent were varied. Optimum conditions were created by applying the extract to a C18 sorbent, washing with methanol–water (35:65, v/v) containing 2% acetic acid, and eluting with a methanol–water mixture with an organic-solvent proportion increasing from 0 to 90%, and containing 2% ammonium hydroxide. With the same sorbent as in the first step, in the second step a mixture of 80% methanol containing 2% ammonium hydroxide was used to completely elute all analytes from the sorbent. Unfortunately, no recovery data are provided in the paper to show the beneficial effect of the second SPE step. LC–ESI(+)-MS of the

purified extract enabled the provisional identification of 49 flavonoids, including several acylated flavonoid-glucosides.

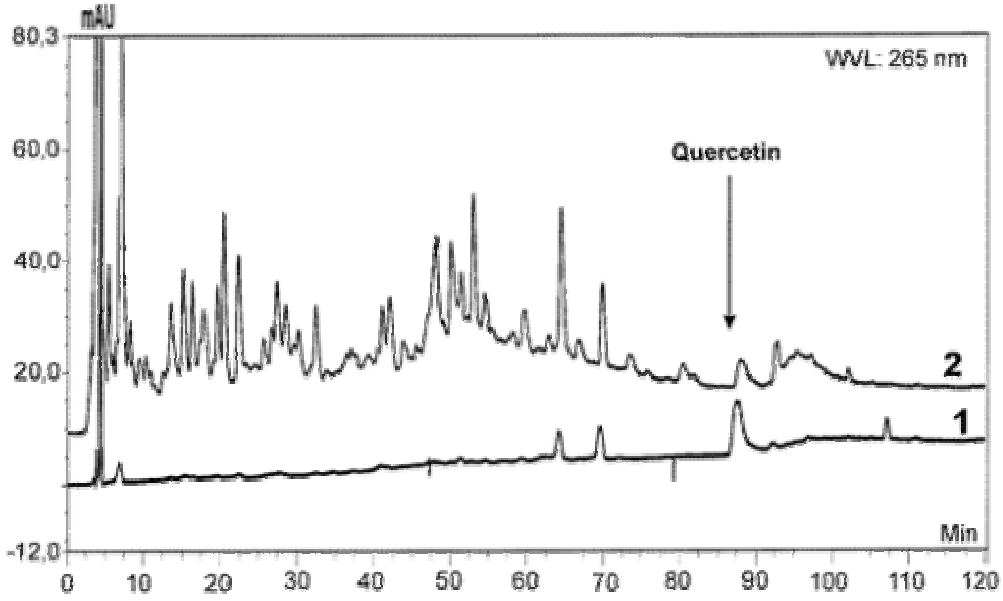


Fig. 3. LC–UV_{265} of Merlot wine without (2) and with (1) molecular imprinted polymer-SPE (elution with acetonitrile) [37].

A relatively new SPE method uses a molecularly imprinted polymer (MIP) as the sorbent. MIPs, typically, are highly selective for the target analyte and usually have good mechanical and thermal stability [37, 38]. A MIP was used to determine quercetin in red wine [37]. The recovery was over 98% when using methanol containing 15% acetic acid or acetonitrile containing 10% aqueous triethylamine as eluent. Fig. 3 shows that use of the MIP greatly reduced the complexity of the LC chromatogram and enhanced the intensity of the quercetin peak. Unfortunately, the MIP was not fully selective for quercetin; structurally related compounds such as morin and (+)-catechin also showed affinity. A general disadvantage of MIP-SPE is that a specific MIP has to be designed for each application and that the method is, in principle, not applicable to other analytes. That is, it can only be used for target analysis and not for screening purposes, while that is the main objective of most flavonoid analyses.

1.2.3 Matrix solid-phase dispersion

MSPD enables the extraction of analytes from samples homogeneously dispersed in a solid support, usually a C18- or C8-bonded silica. In this way, sample extraction and clean-up are carried out simultaneously with, generally, good recoveries and precision. MSPD is frequently used to determine pesticides in *e.g.* fruits, vegetables, beverages and foods [*e.g.* 39, 40, 41, 42], but application to flavonoid analysis [43, 44] was reported only recently. For the LC–

ESI(+)-MS determination of isoflavone aglycones and glycosides in *Radix astragali* – the dried root of *Astragalus membranaceus* that is widely used in Chinese medicine – MSPD was compared to Soxhlet and ultrasonic extraction [43]. For MSPD C18-bonded silica was used and elution was carried out with methanol–water (9:1, v/v). Careful optimization of the eluent composition was needed to prevent co-extraction of interfering matrix components and reduced isoflavonoid yields. For the aglycones, MSPD gave the best extraction efficiency (mean recovery for formononetin, 83%), but for the glycosides Soxhlet gave better results (ononin: UV_{260} peak area ratio Soxhlet/MSPD, 4.3). However, Soxhlet extraction required ten-fold more sample and solvent and the extraction time was much longer. Ultrasonic extraction gave rather poor results (formononetin: UV_{260} peak area ratio ultrasonic/Soxhlet/MSPD, 1/3/4), especially for the aglycones.

A similar MSPD procedure was used to obtain analyte enrichment and sample clean-up for LC–NMR analysis of leaves of red clover [44]. This approach provided sufficiently high concentrations of the seven main isoflavones in these leaves to permit their unambiguous identification while using a mere 500 mg of sample. In this case, MSPD-based sample preparation has the disadvantage that it is somewhat more time-consuming than SE, and therefore more prone to (partial) hydrolysis in the case of flavonoid conjugates. Furthermore, compared with SE the extraction efficiency for the glucosides was found to be lower. Obviously, a systematic study of sorbent materials is urgently needed.

Finally, a study on flavanones and xanthones in the root bark samples of *M. pomifera* should be mentioned [45]. SE, MSPD with a C18-bonded silica and, as a novel approach, MSPD with sea sand were used. For SE, 150 mg dry root bark were soaked in dichloromethane (DCM) or methanol–water (9:1, v/v). For the MSPD procedures 150 mg of dry root bark were mixed with 600 mg of the C18 sorbent or sea sand and 2 ml hexane, packed into a column and eluted with DCM or methanol–water (9:1, v/v). The best results were obtained with the sea sand procedure, with C18-MSPD in second, and SE in last place: when using sea sand, the analyte responses in LC–UV were about 25% higher than with SE. This seems to suggest that, for this application, analyte losses due to incomplete extraction were more important than sample clean-up. In the root bark extracts five prenylated xanthones and two prenylated flavones were found. The LOD of one of these, macluraxanthone, was 3 μg/g. It has to be noted that the authors do not give any information on the sea sand. Apart from its function as a sample disruptor it would be expected to have sorbent properties in order to give better results than C18.

The influence of an MSPD-type treatment on the (partial) hydrolysis of glycosides has not been studied in any of the quoted papers. This is an aspect that requires further attention.

1.2.4 Solid-phase micro-extraction

In SPME a fused-silica fibre coated with polyacrylate or polydimethylsiloxane as a stationary phase is used to extract analytes from a liquid or gaseous sample, or from the headspace above a liquid sample. As is true for SPE, the procedure can effect considerable analyte enrichment. SPME is a straightforward technique and organic solvent consumption is less than in SPE. On the other hand, because it is an equilibrium method, analyte recoveries can be quite low while extraction times frequently are as long as 60 min. SPME is generally combined with GC analysis for the extraction of (semi-)volatile organic compounds from environmental, biological and food samples [46, 47]. SPME has also been coupled with LC to analyze non-volatile and/or polar compounds [46], although this is, in our view, a rather unfortunate and laborious combination and one that is, in the case of flavonoids, not really required because of the many satisfactory alternatives. Nevertheless, two such examples for flavonoid analysis are discussed below [48, 49].

Satterfield *et al.* [48] used SPME to extract genistein and daidzein from human urine in combination with LC–ESI(+)-MS analysis. A Carbowax-templated poly(divinylbenzene) resin proved to be the best fibre type, with a 5-min extraction at pH 4 and a temperature of 35°C. Addition of sodium chloride to aqueous standard solutions of genistein and daidzein gave lower recoveries and caused the formation of sodium ion adducts that interfered in selected reaction monitoring (SRM) ESI(+)-MS. The LODs were 25 and 3 pg/ml for daidzein and genistein, respectively. Concentrations detected in urine 3 h after consumption of 35 g soy protein were 16 ng/ml for both analytes.

To improve the robustness of the SPME procedure, Mitani *et al.* [49] used an open-tubular fused-silica capillary column instead of a fibre. The authors determined the same two analytes in soybean foods using on-line in-tube SPME–LC–DAD UV. An in-tube approach enables automation and usually provides better performance characteristics than manual techniques. Optimum extraction conditions for standard solutions were obtained with 20 draw/eject cycles of 40 μl of sample using a porous-layer open-tubular capillary column; the total extraction-plus-desorption time was 30 min. Analyte recoveries from spiked food were above 97% in all cases. Unfortunately, compared with the earlier study [48], the LODs were about 50-fold higher, *i.e.* about 0.5 ng/ml. In the absence of any further interpretation of the results by the authors of the paper [49], we do not know how to explain the outcome of their study. Admittedly, in the in-tube SPME study an extra hydrolysis step was used since, according to the authors, the hydrophilic glucosides were difficult to adsorb to the capillary and only the aglycones were satisfactorily extracted – but this cannot be considered to account for such a large difference. In the present instance, the poor performance was no real stumbling-block because the concentrations of the aglycones were in the 3–450 μg/g range.

1.3. Separation and detection

The present section will be mainly devoted to LC-based methods (Section 3.1) because these are by far the most important ones in flavonoid analysis (*cf.* Fig. 2). Less common procedures involving GC, CE or TLC, will be discussed in Section 3.2.

1.3.1 Column liquid chromatography

1.3.1.1 General

LC of flavonoids is usually carried out in the reversed-phase (RP) mode, on C8- or C18-bonded silica columns. However, also other phases, such as silica, Sephadex and polyamide are used. Gradient elution is generally performed with binary solvent systems, *i.e.* with water containing acetate or formate buffer, and methanol or acetonitrile as organic modifier. Phosphate buffers are less popular than they used to be, mainly because of the dreaded contamination of ion sources when MS detection is used. LC is usually performed at room temperature, but temperatures up to 40°C are sometimes recommended to reduce the time of analysis and because thermostated columns give more repeatable elution times. If the main aim of the study is to determine the major flavonoids in a sample, run times of 0.5–1 h usually suffice to separate the five to ten compounds of interest [*e.g.* 50, 51]. If, on the other hand, a more exhaustive separation of constituents is intended, run times of up to some 2 h may well be required. Under such conditions, some thirty to fifty compounds can easily be separated (and identified) in a single run, with many conjugates such as glycosides, malonates and acetates frequently being included [*e.g.* 52, 53]. To quote two extremes, when using a special coated silica column, Huck *et al.* [54] needed only 5 min to separate five main aglycones – while a striking exception on the high side is found in a 340-min run for the LC of isoflavones in soy sauces for pattern recognition analysis [55]. Table 3 summarizes some typical examples of LC separation conditions reported in the recent literature. Scrutiny of the text and comparison of the eluent compositions and gradients used in the quoted, and also other, papers reveals that it is often difficult to find out how, and with which main goal, optimization was carried out. Moreover, more recent papers usually do not discuss why elution conditions were selected which differ from those in earlier studies. In several publications, instead of linear gradients, rather complicated gradient profiles are used, comprising several steps and applying various slopes, without any explanation. Obviously, trial-and-error often plays a rather large role. Two exceptions are briefly discussed below.

Table 3. Selection of recent publications on LC analysis of flavonoids.

Method	Eluent	Run time (min)	Sample	Flavonoids	Ref.
LC–UV–ESI-MS/MS	H_2O–MeCN (both with 1% FA)	100	urine	quercetin and kaempferol metabolites	168
LC–UV–APCI-MS/MS	MeCN–H_2O	10	rat serum	naringin, hesperidin, neohesperidin, neoeriocitrin	169
LC–UV–ESI-MS/MS	0.5% aq. $AcONH_4$ –MeOH–MeCN	25	plasma or serum and urine	dietary phytoestogens	170
LC–UV–ESI-MS/MS	0.1% aq. FA–MeOH	52	broccoli	kaempferol, quercetin and isorhamnetin–glucosides and sophorosides	53
LC–UV–ESI-MS/MS	H_2O–MeCN (both with 0.05% TFA)	60	*Cyclanthera pedata*	flavonoid–glucoside and glucoside–malonates	171
LC–UV	H_2O–MeOH (60:40, v/v)	12	*Barosma betulina* leaves and tablets	diosmin and hesperidin	172
LC–UV and LC–ED	aq. FA, pH 2.4–MeCN	26	orange juice	flavanone–glycosides, flavone and flavonol aglycons	173
LC–ED	5 mM aq. $AcONH_4$ –MeCN	80	beer	flavones and phenolic acids	56
LC–UV–ESI-MS/MS and LC–NMR	H_2O–THF–TFA (98:2:0.1, v/v)–MeCN	65	tomato	kaempferol and naringenin–(di) glucosides	174

Stationary phase: in all instances, C18-bonded silica: 3–10 μm d_p, FA, formic acid; $AcONH_4$, ammonium acetate; MeOH, methanol; THF, tetrahydrofuran; TFA, trifluoroacetic acid, MeCN: acetonitrile.

For the analysis of phenolic compounds in beer with LC coupled to electrochemical detection (LC–ED), separation conditions were optimized for a standard mixture of several flavone aglycones and glycosides [56]. Eleven different stationary phases (all C18-bonded silicas) were compared with column dimensions of (100–250) mm × (2.0–4.6) mm ID. The pH and gradient – using water and acetonitrile, with ammonium acetate and formic acid to adjust the pH – were optimized for each column. Acetonitrile was preferred to methanol which often caused a high baseline noise. On the basis of the experimental evidence, four columns were selected: gradient elution was done at pH 3.14 for all of these, but the gradient profiles were slightly different for each of them. Flow rates providing the best resolution and

repeatability varied from 0.23 to 0.9 ml/min, probably because the column IDs varied from 2.1 to 4.6 mm [56]. To our opinion, the variation of too many parameters makes it difficult to reach a proper conclusion regarding the (dis)advantages of the various columns.

Rauha *et al.* [4] studied the influence of the LC eluent composition on the ionization efficiency of five flavonoids in APCI, ion-spray (IS) and atmospheric pressure photoionization (APPI) MS. The effects were found to be considerable. For example, in positive ion (PI) IS and APCI, using 0.4% formic acid as the aqueous component of the LC eluent yielded optimum ionization conditions in contrast with an ammonium acetate buffer of pH 4.0 in the case of negative ion (NI) IS and APCI. The largest effects were obtained for APPI, where pure water gave the best results – with the final choice being *ca*. 5 mM ammonium acetate to create satisfactory LC behaviour of the analytes. With all techniques, the NI mode gave better results than the PI mode, mainly because of lower background noise. Analyte detectability was of the same order of magnitude in all cases, with IS giving marginally better results. The effects of eluent composition on ionization in MS will be further discussed in Section 3.1.3.

According to a recent review [57], stereochemistry is not often discussed in the flavonoid literature. In 1991, a β-cyclodextrin-bonded phase, Cyclobond I, was used in the reversed-phase and normal-phase mode to separate the 2*R* and 2*S* diastereomers of flavanone glycosides and benzoylated flavanone glycosides [58]. Other papers discussed the enantiomeric separation of flavanones [59] and the diastereomeric separation of flavanone–glycosides [60].

1.3.1.2 Detectors in LC

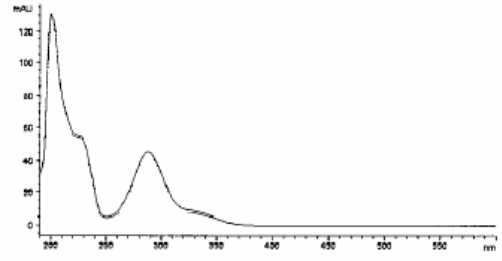
UV absorbance detection. All flavonoid aglycones contain at least one aromatic ring and, consequently, efficiently absorb UV light. The first maximum, which is found in the 240–285 nm range, is due to the A-ring – and the second maximum, which is in the 300–550 nm range, to the substitution pattern and conjugation of the C-ring [61]. Simple substituents such as methyl, methoxy and non-dissociated hydroxyl groups generally effect only minor changes in the position of the absorption maxima. Already several decades ago, UV spectrophotometry was, therefore, a popular technique to detect and quantify flavonoid aglycones. More recently, UV detection became the preferred tool in LC-based analyses and, even today, LC with multiple-wavelength or diode-array (DAD) UV detection is a fully satisfactory tool in studies dealing with, *e.g.*, screening, quantification of the main aglycones and/or a provisional sub-group classification (*cf.* Fig. 1).

It will be clear that what has been said above for the detection and characterization of the aglycones, is also true for their conjugates. Generally speaking, this facilitates the recognition of so-called satellite sets, comprising aglycones, their glycosides, glycoside–malonates and, in some cases, glycoside–acetates (see Fig. 4). Unfortunately, most glycosides

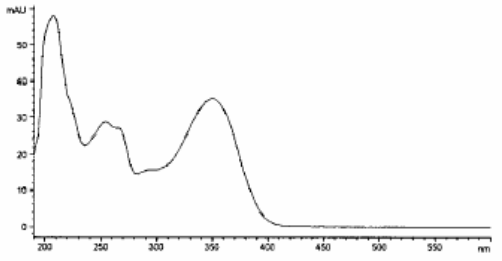
and acyl residues are poor chromophores; consequently no further distinguishing can be achieved by means of DAD UV detection. As regards the potential and limitation of satellite-set recognition, Fig. 4 shows that – while even the identification of two flavonoids from the same sub-class, the isoflavones – does not present a real problem, the UV spectra of the various members of each set (peaks 1–3, 7 and 4–6, 8) are mutually indistinguishable.

Characteristic UV spectra of four of the main classes of flavonoids are shown in Table 4. Details on wavelengths of maximum absorption and molecular extinction coefficients, ε, for over 150 flavonoids can be found in an early work by Mabry *et al.* [61]. The general experience is that the spectra in ethanol, methanol and acetonitrile are essentially the same and that log ε_{max} values of the main absorption band are on the order of 3.4–4.6. The spectra included in Table 4 and the λ_{max} data summarized in Table 4 clearly indicate that (i) the various flavonoid sub-classes can indeed be provisionally distinguished from each other, *i.e.* that LC–DAD UV is an interesting complementary tool during structural characterization (see Section 4), and (ii) a limited number of monitoring wavelengths suffices for a general flavonoid screening: flavonoid detection is usually carried out at 250, 265, 290, 350, 370 and/or 400 nm (with an added wavelength in the 500–525 nm range if anthocyanidins are included [57]). The modest losses of analyte detectability caused by the selection of less than fully optimized detection wavelengths are generally considered acceptable. Limits of detection (LODs) down to 1–10 ng (injected mass) are repeatedly reported [62, 54]. This implies that, for an injection volume equivalent to 1 g or 1 ml of original sample, concentration LODs in the low ng/g range can be obtained. In real samples often much higher concentrations are encountered for the most abundant – and, consequently, most relevant – target aglycones (see Table 5), and analysis will not be too demanding.

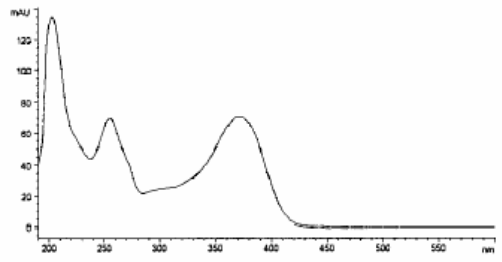
Table 4. λ_{max} of some representative flavonoids of four of the main sub-classes, with examples of each class [20]*.



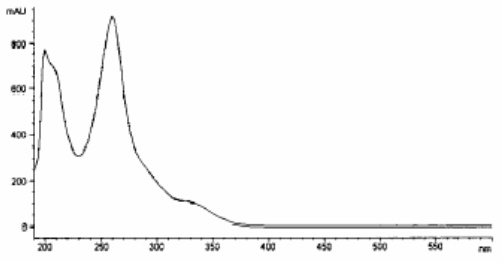
hesperetin



luteolin



quercetin



genistein

Flavonoid	λ_{max}
Flavones	
5-Hydroxyflavone	272, 337
Apigenin	268, 337
Luteolin	253, 347
3-Methylquercetin	255, 355
Flavonols	
Fisetin	253, 370
Morin	263, 380
Quercetin	250, 370
Isoquercitrin	258, 360
Myricetin	255, 378
Isoflavones	
Genistein	260, 328 (sh)
Biochanin A	261, 326 (sh)
Daidzein	250, 302 (sh)
Formononetin	249, 302
Flavanones	
Naringin	284, 330 (sh)
Naringenin	288, 325 (sh)
Hesperetin	289, 330 (sh)

* See Figure 1; data taken from refs. [175, 99, 3].

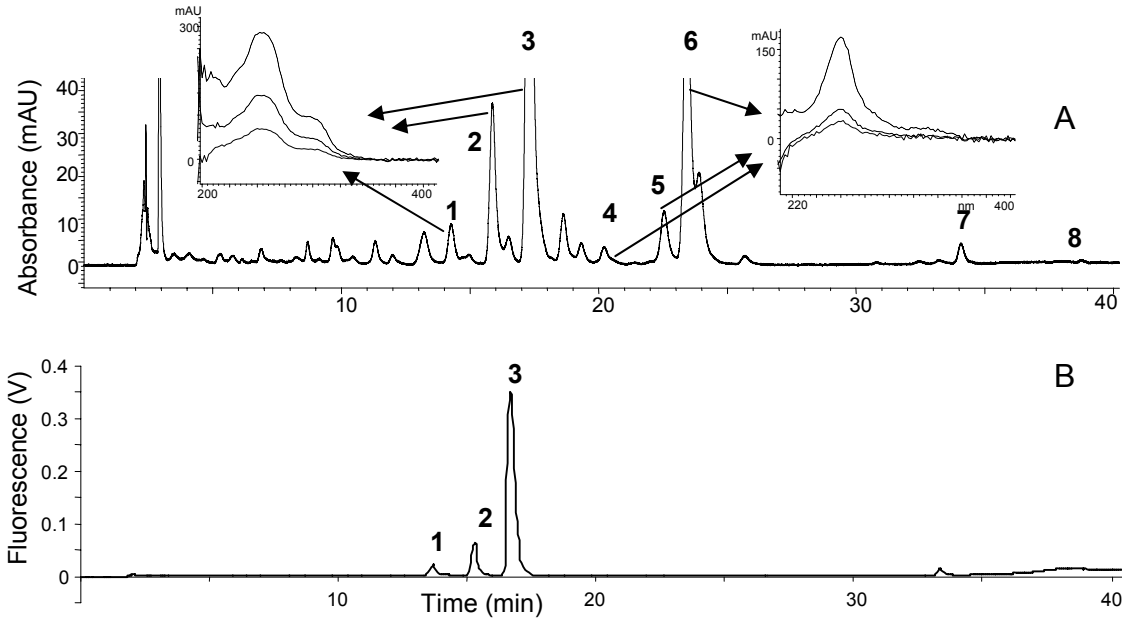


Figure 4. (A) RPLC–UV_{250} and (B) RPLC–FLU (ex, 250 nm; em > 450 nm) of an extract of *T. pratense* leaves. Peak numbering: (1) an isomer of FGM, (2) formononetin–7-*O*-β-D-glucoside, (3) formononetin–7-*O*-β-D-glucoside–6"-*O*-malonate (FGM), (4) an isomer of BGM, (5) biochanin A–7-*O*-β-D-glucoside, (6) biochanin A–7-*O*-β-D-glucoside–6"-*O*-malonate (BGM), (7) formononetin and (8) biochanin A. For further explanation, see text; the isomers were later identified using LC–NMR, see Section 4.3 [98].

In order to illustrate the general usefulness and application of LC–(DAD) UV the analysis of an *Helichrysum stoechas* extract [63] is presented in Fig. 5. The DAD UV spectra shown as inserts A–M illustrate the widely differing spectral characteristics of various flavonoid subclasses. The spectra played an important role in the identification: for instance, peaks G and H have the same aglycone skeleton (naringenin). Based on the mass spectrum (also recorded) peak J could either be a kaempferol or a luteolin conjugate, but the DAD UV spectrum was only consistent with the former. A similar study was reported on flavones and isoflavones in a *Genista tinctoria* extract [64].

Fluorescence detection. In flavonoid analysis, fluorescence detection is used only occasionally, because the number of flavonoids that exhibit native fluorescence is limited. For these compounds, LODs in LC and CE are typically about an order of magnitude lower than with UV detection. Moreover, their fluorescence facilitates selective detection in complex mixtures [65]. Classes of flavonoids that show native fluorescence include the isoflavones [66], flavonoids with an OH group in the 3-position, *e.g.* 3-hydroxyflavone [67] and catechin [*trans*-3,3′,4′,5,7-pentahydroxyflavan] [68], and methoxylated flavones, *e.g.* 3′,4′,5′-trimethoxyflavone [69]. As an example, Fig. 6 illustrates the selectivity of fluorescence detection for the determination of 3′,4′,5′-trimethoxyflavone in an extract of *Flos primulae veris*. Fluorescence detection (LOD (S/N=3), 25 μg/l) was 10-fold more sensitive than UV detection. However, with MS in the selected ion monitoring (SIM) mode, detectability was

even better (LOD, 5 μg/l) [69]. When fluorescence detection is used in combination with UV it offers the possibility to discriminate between fluorescent and non-fluorescent co-eluting compounds [70, 65].

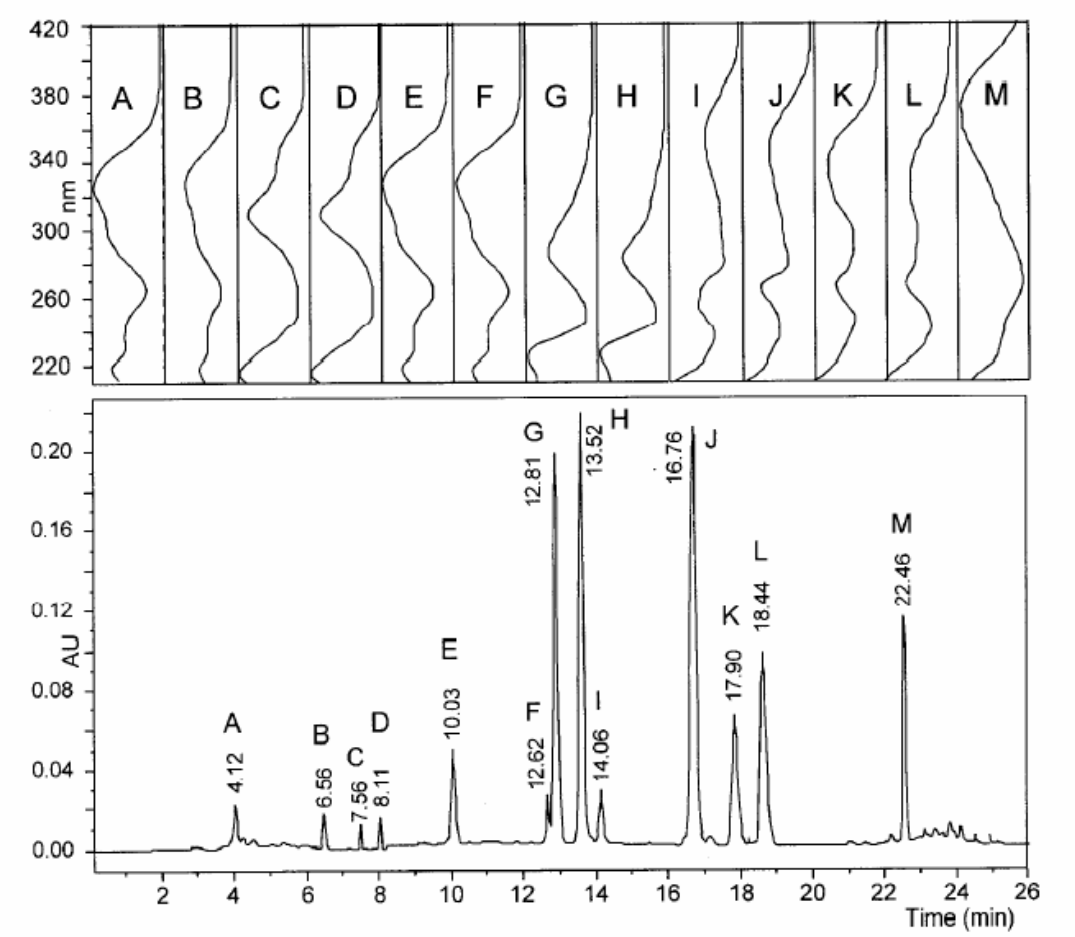


Fig. 5. LC–DAD UV of an *H. stoechas* extract ((λ = 270 nm) with the spectra recorded at the peak apices as inserts A–M [63].

The nature of the functional groups and their substitution pattern determine whether a particular flavonoid is fluorescent or not. For example, from amongst the isoflavones, only those that do not have an OH group in the 5-position show strong native fluorescence – as is true for compounds 1–3 and 7 in Fig. 4B. Such compounds exhibit large Stokes' shifts, possibly due to a change of the molecular structure of the molecule from non-planar in the S_0 state to planar in the S_1 state, with an accompanying change in electric dipole moment. These large shifts create a high selectivity over impurity fluorescence from the matrix, since it enables the use of long emission wavelengths for detection [66]. The large Stokes' shifts for formononetin and its glucoside, ononin, are shown in Fig. 7. Interestingly, the fluorescence excitation and, though less so, emission spectra of formononetin also show a pH dependence; an extra band at 340 nm starts to come up at pH 6, the pK_a value of the analyte. At higher pH the compound is predominantly in its anionic form. The shift in emission is much less

pronounced, *i.e.* from 495 nm for the neutral molecule to 470 nm for the anion. As expected, for ononin no such effect is observed since the glucose substituent prevents ionization.

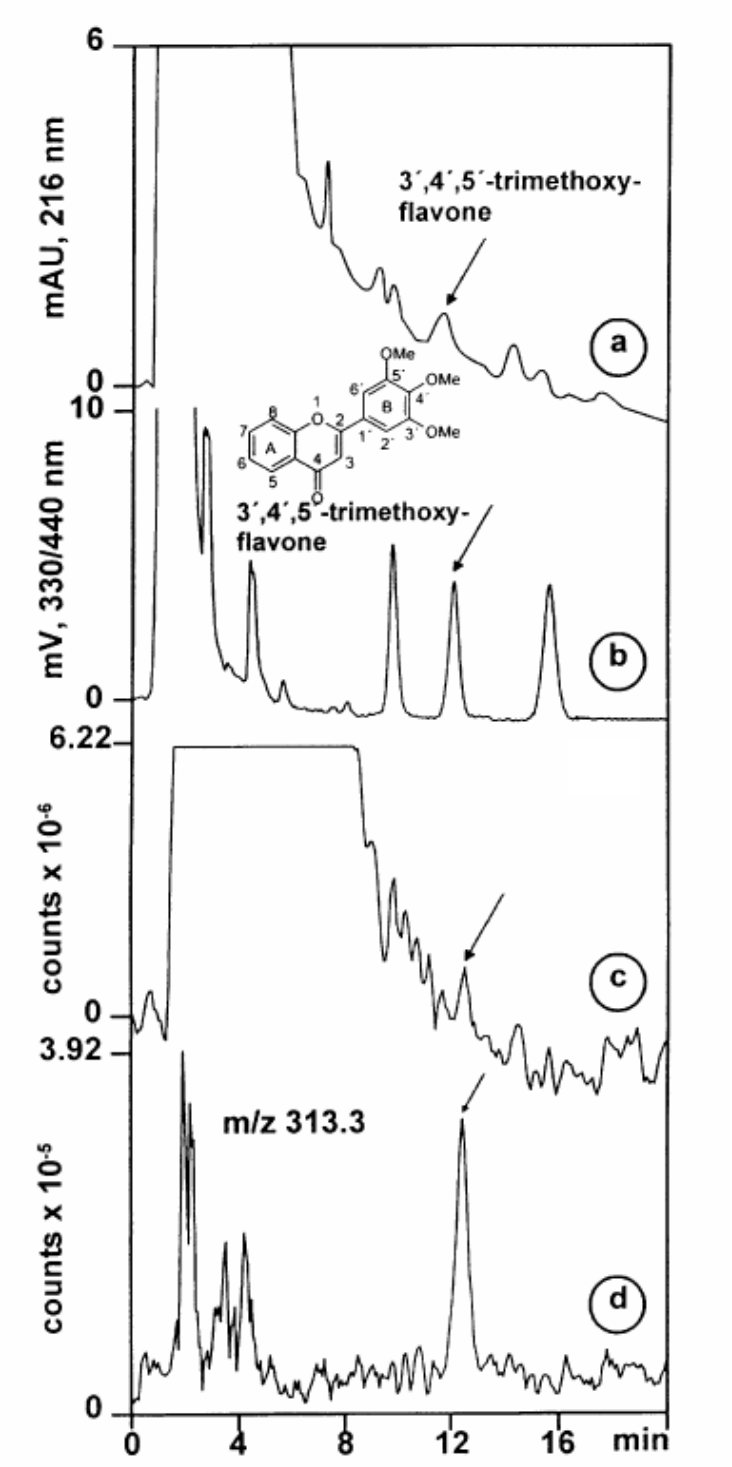


Fig. 6. Comparison of detection methods for 3′,4′,5′-trimethoxyflavone in an extract of *Flos primulae veris*: (a) RPLC–$UV_{216,}$ (b) RPLC–FLU (ex, 330 nm; em, 440 nm) and RPLC–ESI(+)-MS in (c) full-scan and (d) extracted ion chromatogram of *m/z* 312–314 [69].

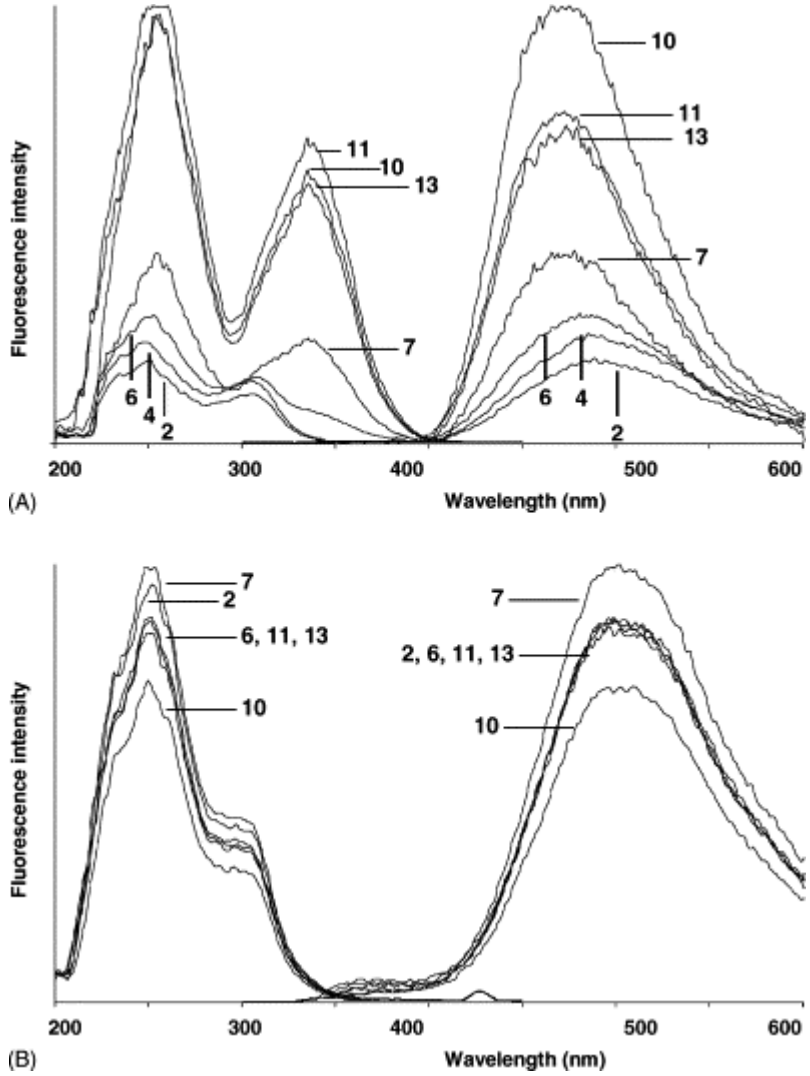


Figure 7. Fluorescence excitation and emission spectra of (A) formononetin and (B) ononin in methanol–water (1:1, v/v) at the pH values indicated [66].

Table 5 Selected examples of flavonoid concentrations in several sample types.

Sample	Flavonoids	Conc. (g/kg)	Ref.
Orange, lemon and grapefruit juices	hesperidin, naringin, neohesperidin, quercetin	0.005 – 2.0	176
Apple peel and pulp	catechin, rutin, procyanidin B2	0.0001– 0.0007	177
Grape berry peel	rutin, quercitrin, quercetin	0.2 – 2.0	178
Black, green and jasmine tea infusions	epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate	0.01 – 0.15	121
Dalbergia odorifera (Chinese medicine)	4′-methoxy-2′,3,7-trihydroxyisoflavanone, liquiritigenin, melanettin, violanone, vistitone, formononetin, dalbergin, sativanone, medicarpin	0.005 – 0.03	99
St. John's Wort powder and tablets	rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin	0.3 –30.0	52
Red clover	daidzin, daidzein, genistin, genistein, formononetin, ononin formononetin–glucoside–malonate, biochanin A, sissotrin, biochanin A–glucoside–malonate	0.04 – 5.0	65
Scutellaria baicalensis roots	Baicalein–7-*O*-glucuronide, wogonin–7-*O*-glucuronide, baicalein, wogonin	0.07 – 5.0	179

As stated above, some flavonols (*cf.* Fig. 1) also show native fluorescence. Here, the 3-OH group is involved in excited-state intramolecular proton transfer, which causes solvent-dependent dual emission, *i.e.* two emission bands show up of which the ratio depends on the solvent composition. This phenomenon has been studied extensively in the literature [see *e.g.* 67, 71, 72] and will not be further discussed here.

To extend the application range of fluorescence detection, derivatization has been used. For example, quercetin, kaempferol and morin, with their 3-OH, 4-keto substituents, can form complexes with metal cations, some of which are highly fluorescent [73, 74]. Hollman *et al.* [73] used post-column derivatization for the determination of quercetin and kaempferol, based on the formation of fluorescent complexes with Al(III). LODs were found to be 0.15 and 0.05 ng/ml for quercetin and kaempferol, respectively. The method was used to study the bioavailability of quercetin from onions and apples in humans [75]. Plasma levels of quercetin of nine individuals were measured over a 36-h period. Peak levels in the plasma were reached within 0.7 h after ingestion of onions (220 ng/ml), 2.5 h after ingestion of apples (90 ng/ml) and 9 h after ingestion of quercetin rutinoside (90 ng/ml).

Electrochemical detection. Since most flavonoids are electroactive due to the presence of phenolic groups, electrochemical detection (ED) can also be used. Although ED is not as sensitive as fluorescence detection, LODs can be quite low: for *trans*-resveratrol in rat blood an LOD of 2 μg/l was obtained using LC–multichannel-ED [76]. In a recent paper isoflavones in soybean food and human urine were determined by LC–coulometric array-ED [77]. LC–UV–MS was used for identification purposes. The coulometric electrode array detector consisted of a 6-μl flow-through analytical cell containing an Ag/AgCl reference electrode, a platinum wire counter-electrode and eight porous graphite working electrodes (carbon paste). For standard solutions of daidzein and genistein the highest signal was found at 450 mV, most likely corresponding to an oxidation signal. Calibration plots were linear for genistein but not for daidzein, probably due to saturation of the electrode surface by the analyte. Under optimum conditions (eluent: acetonitrile–acetate buffer) the LODs for daidzein and genistein were 400 pg/ml. In several soybean foods, the concentrations of daidzein and genistein were 20–200 and 60–300 μg/g, respectively (recoveries, 95–107%). In human urine, only the corresponding glucosides, daidzin and genistin, were found; their concentrations were *ca.* 5 μg/g.

Peyrat-Maillard *et al.* [78] used RPLC–ED to evaluate the anti-oxidant activity of phenolic compounds, including eleven flavonoids, by measuring the accelerated auto-oxidation of methyl linoleate in anhydrous dodecane, under strongly oxidizing conditions. For all flavonoids, two maxima showed up in the peak area *vs.* potential voltammograms, as shown for rutin in Fig. 8. The first maximum corresponds to the oxidation of the phenolic

substituents on ring B, the second one probably comes from the other less oxidizable phenolic groups. For the flavonoids there was no clear linear relation between the anti-oxidant activity and the ED signal, since various structural parameters play a role. For instance, glycosylation of the 3-hydroxyl group decreases the antioxidant or radical-scavenging activity in flavonols, but not their electrochemical behaviour. The decrease can be attributed to the steric hindrance posed by the glucose group or to poor solubility in the alkane solvent used in these experiments. However, for most analytes, the antioxidant efficiency could be related to the value of the first maximum which corresponds to the lowest energy required to donate an electron.

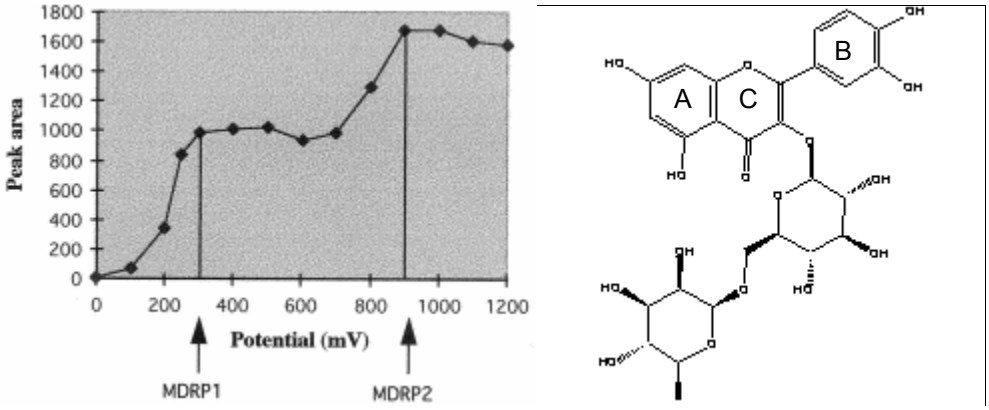


Fig. 8. Voltammogram and structure of rutin. MDRP: maximal detector response. Modified from [78].

1.3.1.3 LC–MS

In flavonoid analysis, MS is the state-of-the-art detection technique in LC. In this section we confine ourselves to single-stage MS. Table 6 summarizes relevant information on a selection of recent LC–MS studies. In most cases single-stage MS is used in combination with UV detection to facilitate the confirmation of the identity of flavonoids in a sample with the help of standards and reference data. For the identification of unknowns, tandem mass spectrometry (MS/MS or MS^n) is used – a technique that deserves a separate discussion which is presented in Section 4.2. It should be noted that six of the reviews in Table 1 are solely dedicated to MS. In two of these the main focus is on structural characterization [57, 79], and in four on the application of LC–MS in flavonoid analysis [80–83].

In the LC–MS of flavonoids – as in many other application areas – atmospheric pressure ionization interfaces, *i.e.* atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are used almost exclusively today. Both positive (PI) and negative (NI) ionization are applied. ESI is more frequently used in flavonoid analysis, but APCI is gaining in popularity and in some cases better responses are obtained in that mode [84–86]. According to most studies, for both APCI and ESI the NI mode provides best

sensitivity. However, the PI mode should not be neglected, since useful complementary information is often obtained in studies dealing with the identification of unknowns. For the rest, one should be aware that, with all four modes of operation, analyte responses can vary considerably – and rather unexpectedly – from one sub-class to another, and even within one class [84, 4]. In addition, the composition of the LC (gradient) eluent, its pH and the nature of the buffer components added, can have a distinct influence [*e.g.* 84, 87], as was discussed in Section 3.1.1 for a study by Rauha *et al*. [4]. In flavonoid analysis, the most common additives are acetic acid [27], formic acid [88], ammonium acetate and ammonium formate [89, 84]. Trifluoroacetic acid has also been used [90] although it is known to suppress the ionization due to ion-pairing and surface-tension effects.

The mass spectra of flavonoids obtained with quadrupole and ion-trap instruments typically are closely similar, even though relative abundances of fragment ions and adducts do show differences [84]. Therefore, direct comparison of spectra obtained with these two instruments is allowed. The main advantage of an ion-trap instrument is the possibility to perform MS^n experiments, which enables the confirmation of proposed reaction pathways for fragment ions [84].

Table 6. Selected examples of the use of LC–MS for flavonoids analysis.

Sample	Flavonoids	Ionization mode	Conc. (mg/g)	LC eluents	Ref.
Genista tinctora	16 flavone– and isoflavone–glycoside(–malonate)s and aglycons	ESI(–)	15–0.003	MeCN, AcOH	180
Leguminosae (four species)	isoflavone– and flavonol–glucoside–(di)malonates and flavonol (di)glycosides	APCI(–)	0.03–65	MeOH, ammonium formate	98
Scutellaria baicalensis	wogonin–5-*O*-glucoside, wogonoside, baicalin, wogonin, norwogonin, chrysin–6-*C*-arabinose-8-*C*-glucose, chrysin–6-*C*-glucose-8-*C*-arabinose	ESI(–)	-	MeCN, $AcONH_4$	181
Ginkgo biloba tablets	rutin, quercitrin, quercetin, kaempferol, isorhamnetin, quercetin–glycoside	ESI(–)	5–330 μg/tablet	MeCN, FA	182
Hypericum perforatum, Rhodiola rosea, red grape wine, orange juice, green tea	50 flavonols, flavanones, flavones, catechins and anthocyanins	ESI(+)	0.0002–0.01	MeOH, FA	183
Red clover	49 isoflavone–glucoside–malonates and –acetates, glycosides and aglycons	ESI(+)	-	MeCN, H_2O	3
Helichrysum stoechas	6 chalcones, flavonols and flavanones	APCI(±)	1.5–3	MeCN, ammonium formate	63

ammonium acetate: $AcONH_4$, acetic acid: AcOH, MeOH: methanol, FA: formic acid.

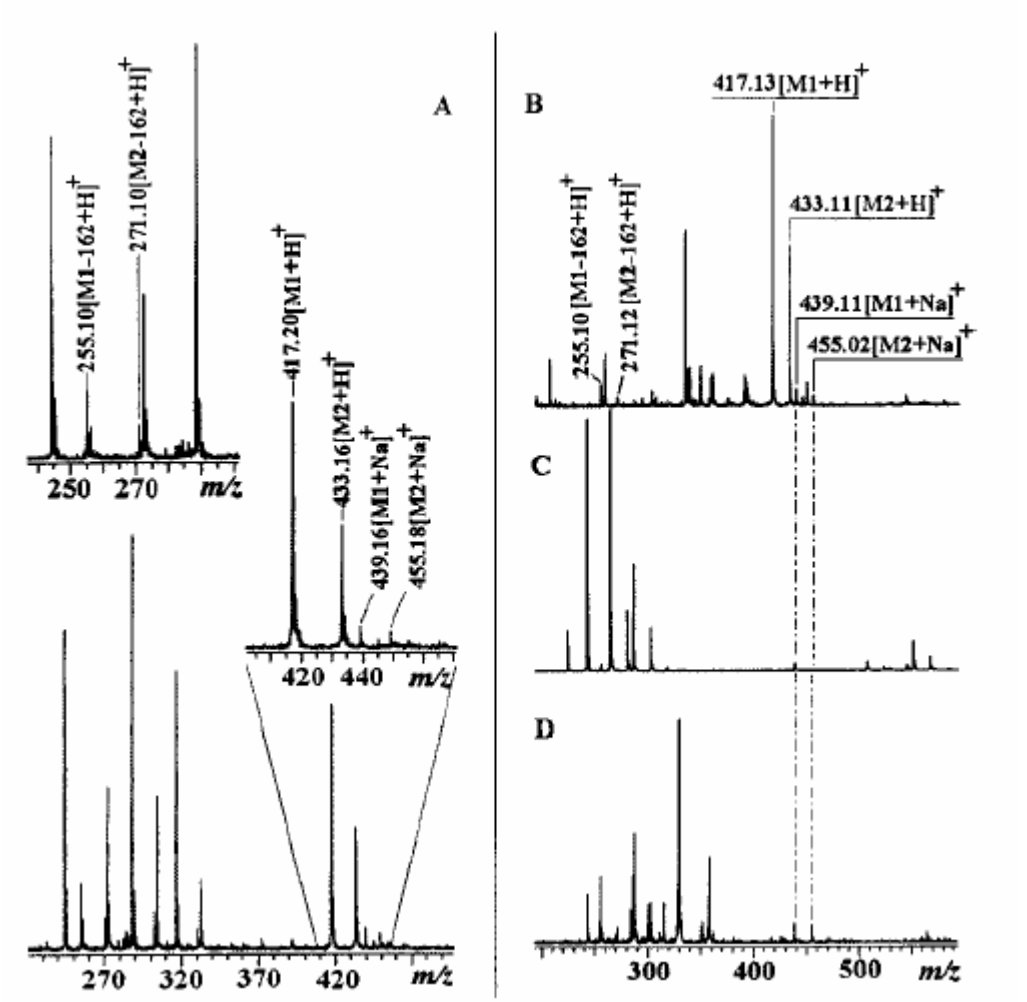


Fig. 9. MALDI(–)-TOF MS and the performance of four MALDI-TOF MS matrices: A, 2,5-dihydroxybenzoic acid (DHB); B, 2,4,6-trihydroxyacetophenone (THAP); C, 2-(4-hydroxyphenyl-azo)benzoic acid (HABA); D, 3-aminoquinoline (3-AQ), for the isoflavones: M1, daidzin (6.3×10^{-4} M in 70% methanol) and M2, genistin (6.7×10^{-4} M in 70% methanol) [95].

Next to ESI and APCI, ionization techniques such as electron ionization (EI) [91], chemical ionization (CI) [92], fast atom bombardment (FAB) [93], and matrix-assisted laser desorption ionization (MALDI) [94–96] are also used. The potential of off-line MALDI-TOF MS for flavonoid analysis was explored recently for flavonoids in red wine and fruit juices [94], soy products [95], and onions and green tea [96]. Samples were pre-treated with SPE and preparative LC and the collected flavonoid- and anthocyanin-containing fractions were analyzed by MALDI-TOF MS. Of the various MALDI matrices tested, 2,4,6-trihydroxyacetophenone (THAP) proved to be the best for flavonol–glycosides from red wine and fruit juices and for anthocyanins from onions and tea, whereas 2,5-dihydroxybenzoic acid (DHB) was the preferred matrix for isoflavones from soy products. Fig. 9 shows MALDI-MS spectra of daidzin and genistin, recorded with four different matrices. In the spectra of all flavonoids, the only ions detected were the protonated molecules and the minor sodium and

potassium adducts; for the glycosides, glycosidic cleavage was observed. Contrary to studies of flavonoid–glucoside–malonates with ESI- and APCI-MS [44, 97], in the MALDI-TOF mass spectra of the glucoside–malonates in the soy samples, no loss of the malonate moiety was found. Sample clean-up with SPE was invariably needed because the sample matrix components with masses between 700 and 900 Da suppressed flavonoid ionization. Anthocyanins in juice extracts showed a linear response with concentration in the range of 1 to 10 mg/l, but rather unexpectedly the relative responses of the anthocyanin–diglucosides were four times lower than those of the monoglucosides. According to the authors, quantification is possible by using an internal standard.

In LC–MS, sets of flavonoids with the identical aglycon mass and which comprise, *e.g.* the glucoside–malonate, the glucoside and the aglycone – also called satellite sets – are easily recognized. In *T. pratense*, for example, satellite sets of the main isoflavones formononetin and biochanin A were found, which consisted of the glucoside–malonate, an isomer, the glucoside and the aglycone. Thirteen of such satellite sets were found, comprising 40 isoflavones. It lies at hand to conclude that, when screening a plant extract for unknowns, knowledge of the presence of satellite sets [98] simplifies target analysis. However, a note of warning should be added. The literature shows that, even within the same plant species, various authors find different flavonoid conjugates. For example, in the paper cited above mainly glucoside–malonates were reported, whereas in another study on flavonoids in *T. pratense*, also several glycoside–acetates were found [3]. In an LC–APCI(–)-MS study of four leguminous plant species, the flavonoid patterns were found to be widely different: in *T. pratense* L. and *T. repens* L., the main constituents were flavonoid glucoside–(di)malonates, while *T. dubium* L. and *L. corniculatus* L. mainly contained flavonoid (di)glycosides. Remarkably, those observations markedly differed from those reported in other papers, which sometimes also differed from each other [99, 3]. This may be (partly) due to differences in environmental factors, growth conditions, etc. – an aspect that, until now, has not been studied in any detail [98].

The structural characterization of flavonoid–glycosides by means of LC–MS/MS will be discussed in Section 4.2.4.

1.3.2 Less common methods

1.3.2.1 General

In this section three separation techniques will be considered, *i.e.* GC, CE and TLC, which are used less frequently than LC. The renewed attention for classical techniques such as GC and TLC can be called somewhat surprising. On the other hand, CE is a relatively novel technique, and has only been used for flavonoid analysis in the last ten years. Techniques like high-speed

counter-current chromatography (HSCCC) and supercritical fluid chromatography (SFC) are not considered here. Their role in flavonoid analysis is limited and they are both discussed quite elaborately in the 2004 review of Tsao and Deng [100], which deals with separation techniques for phytochemicals.

1.3.2.2 Gas chromatography

Gas chromatography was used for the analysis of flavonoids already in the early 1960s. In the first paper on this topic [101], derivatized flavonoids were separated on a semi-preparative scale using a SE-30 silicone polymer column with subsequent thermal conductivity detection; fractions were collected for IR and UV-vis spectroscopy. After the introduction of LC, GC analysis of flavonoids became less prominent, but recently it received renewed attention [*e.g.* 102–104], possibly because of the developments in high-temperature GC and the introduction of improved derivatization procedures – topics that will be discussed below. However, most recent GC studies use conventional temperature programmes and derivatization methods.

GC-based methods provide high resolution and low detection limits, but they are labour-intensive because derivatization – in most cases directed at the formation of trimethylsilylether (TMS) derivatives – is unavoidable to increase the volatility of the flavonoids and to improve their thermal stability. It should be noted that for flavonoids with more than one hydroxyl substituent methylation may yield several derivatives, which makes quantification difficult. The separation conditions have not changed much since the early 1960s although, today, fused silica capillary columns are used instead of packed glass columns.

Most recent papers on the GC analysis of flavonoids are in the biological and nutritional area, and focus on the flavonoid antioxidant activity, metabolism or taxonomy. Little attention is paid to method development and, in our opinion, LC would have been a better choice in many cases. Typically in GC, flavonoids are hydrolyzed and converted into their TMS derivatives, injected onto a non-polar DB-5 or DB-1 column in the split or splitless mode and separated with a linear 30–90 min temperature programme up to 300°C. *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and *N*-(*tert*.-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS) are the most commonly used derivatizing agents, and EI-MS in the selected ion monitoring (SIM) mode with a source temperature of up to 250°C is often used for detection. The molecular ion, $[M+H]^+$, and fragments formed by the loss of CH_3 and/or CO and retro-Diels–Alder reactions are typically used for detection. The MS fragmentation is discussed in Section 4.2. In Table 7 relevant information on recent GC studies is summarized; a representative selection is discussed below.

Table 7. Recent publications on GC analysis of flavonoids.

Method	Derivatization	Sample	Flavonoids	LOD (mg/l)	Ref.
HRGC–MS and –FID	TMS	standard mixture	hesperidin, chrysin, apigenin, quercetin	12.5–50	184
GC–MS	in-vial TMS derivz. + extrn.	various herb extracts	naringenin, galangin, kaempferol, luteolin	20–400	104
GC–MS	TMS	*Ginkgo biloba*	kaempferol, quercetin, isorhamnetin	0.5–2.5	103
HT–HR GC–MS	none; before GC, sample subjected to prep. LC	*Vellozia graminifolia*	6 mono-isoprenylated flavonoids	n.d.	110
GC–MS	with and without TBDMS	*Populus tremuloides*	4',5-dihydroxy-7-methoxyflavanone, 4',5,7-trihydroxyflavanone, 3,5,7-trihydroxy-4'-methoxyflavone 3,4',5,7-tetrahydroxyflavone	n.d.	185
HT–HRGC –MS and –FID		*Loncho-carpus urucu*	several flavonoids	0.5 (rotenone with FID)	109
GC–MS		propolis	pinostrobin chalcone, pilloin pinocembrin, tectochrysin, genkwanin, chrysin, galangin, 5-hydroxy-4',7-dimethoxyflavone	n.d.	135
GC–MS	TBDMS	fruits and nuts	daidzein, genistein	LOQ: 1 μg/kg	105
Isotope dilution GC–MS	TMS	serum and soy foods	daidzein, genistein	n.d.	102
GC–MS	BSTFA (TMS)	human urine	daidzein, genistein, biochanin A, formononetin	10–23	186
GC–MS	BSTFA (TMS)	human and rat serum plasma and urine	quercetin, catechin	0.1–1	187

TMS, trimethylsilyl; n.d., not determined.

To determine the genistein and daidzein contents of fruits and nuts, freeze-dried samples were extracted with methanol and hydrolyzed with cellulase in acetate buffer. The aglycones were extracted with ethyl acetate, derivatized with TBDMS and subjected to GC–MS [105]. Of the eighty samples, only 37 contained detectable amounts of the isoflavones, of which nine contained more than 100 μg/kg wet wt. The limit of quantification was 1 μg/kg.

An improved derivatization procedure used in-vial derivatization–extraction for the GC–MS analysis of flavonoids and phenolic acids in various herbs [104, 106]. Derivatization takes place under basic conditions so that the hydroxyl groups of the analytes will be deprotonated. The anionic nucleophiles are transferred to the organic phase as ion-pairs using a phase-transfer catalyst (PTC) and are next subjected to reaction with methyl iodide. Polymer-bound tri-*n*-butylmethylphosphonium chloride proved to be the best PTC. In the SIM

mode, the LODs of the flavonoids in the extracts were 4–40 ng/ml. The GC–MS chromatogram of a *Mentha spicata* extract is shown in Fig. 10.

Several recent papers use GC–MS to determine flavonoids in food and food supplements – such as soy products and fruit – and in serum to study the bioactivity and bioavailability of flavonoids and phyto-estrogens [*e.g.* 102, 105, 107 and 108]. An interesting aspect of the first paper cited is that isotope dilution GC–MS was used [102]. At a first glance, there seems to be hardly any challenge from an analytical point of view since the analyte concentrations are high in soy; the daidzein and genistein concentrations are even in the low mg/g range. However, after consumption of soy food only low levels of these isoflavones are found in the human body and the challenge is to determine such low concentrations and accurately correct for losses during the various sample-treatment steps. To give an example [102], for the determination of phyto-estrogens in human serum, samples were hydrolyzed with β-glucuronidase, the aglycones were extracted with ethyl acetate and the phyto-estrogen fraction isolated on a Sephadex LH20 column, with subsequent derivatization with BSTFA. Deuterated internal standards were used for the isotope dilution procedure, with SIM-mode detection. The concentrations of daidzein and genistein varied between 2 and 900 ng/ml (means, 80 ng/ml and 160 ng/ml, respectively) for 42 human serum samples. A lack of analytical performance data prevents further evaluation.

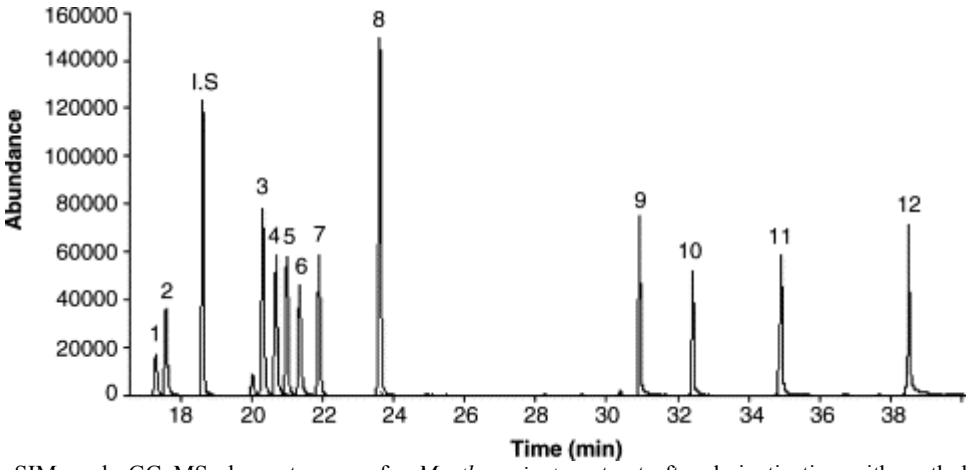


Fig. 10. SIM-mode GC–MS chromatogram of a *Mentha spicata* extract after derivatization with methyl iodide. Flavonoid peak assignment: 9, naringenin, *m/z* 300; 10, galangin, *m/z* 311; 11, kaempferol, *m/z* 327; 12, luteolin, *m/z* 328; the other peaks are phenolic acids [104].

In conventional GC it is very difficult to analyze flavonoid glycosides even after derivatization. Therefore, Pereira *et al.* [109] used high-temperature–high-resolution (HT–HR) GC–MS, with columns that can withstand temperatures up to 400°C, for the glucoside hesperidin. Unfortunately, the LOD of hesperidin in a standard solution was found to be as high as 50 mg/l with both cold on-column injection and splitless injection at 370°C. Other

disadvantages were that derivatization of hesperidin with BSTFA took 72 h before analysis and that the derivative showed severe peak tailing. Interestingly, HT–HRGC–MS with cold on-column injection has been used without derivatization to determine mono-isoprenylated flavonoid aglycones, in an extract of *Vellozia graminifolia* [110]. After fractionation by means of preparative LC and TLC screening, 'positive' fractions were combined and analyzed by HT–HRGC–MS. Six mono-isoprenylated flavonols were identified on the basis of their melting points and their MS, IR, ^{13}C- and ^{1}H-NMR spectra.

It will be obvious that, for the analysis of flavonoids, GC will not easily replace LC – and certainly not if emphasis is on both aglycones and glycosides. For such studies, derivatization is needed (and several derivatives may be formed for one analyte), even in HT–GC. More efficient in-vial derivatization and the low LODs of SIM-mode MS detection are interesting advantages. However, they clearly do not outweigh the rapidity of direct LC–MS(/MS) procedures and the possibility to easily screen samples for target analytes as well as unknowns.

1.3.2.3 Capillary electrophoresis

Most studies that use CE for the analysis of flavonoids are in the field of natural product research, including the analysis of plants [111–113], vegetables [114], herbs [115] and other plant- or fruit-derived products [116, 12, 117]. The CE modes primarily used are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) with, typically, a phosphate or borate buffer, capillaries of 50–100 μm I.D., voltages of 10–30 kV and 10–50 nl injection volumes. Detection is usually performed with UV, but also fluorescence [111], ED [115, 118] and MS detectors are used [117, 119].

From amongst the reviews on flavonoid analysis quoted in Table 1, one is entirely devoted to CE [120], while three reviews cover CE techniques in a more general way [62, 100, 121]. In two of the latter three papers [62, 100] only limited attention is devoted to CE and most of the cited references are for pre-2000 publications. The discussions are of a rather general nature, mentioning well-known aspects such as the on-going technical developments, the possibility to use very small samples, and the absence of any detection advantages over LC. The other two reviews are also limited to pre-2000 papers, with one of these discussing the merits and de-merits of CE–MS, and the other one focusing on quantification and method validation. For reasons of complementarity, we will pay attention primarily to the 2000–2004 literature. The focus will be on the analytical procedures and a comparison of the modes of operation; a representative selection of the papers published in the last five years is summarized in Table 8.

Table 8. Representative studies on CE of flavonoids.

Method	Eluents (v/v)	Sample	Flavonoids	Ref.
CE–ED	50 mM borate buffer (pH 9.2)	*Flos chrysanthemum*	acacetin, hesperetin, kaempferol, apigenin, luteolin, quercetin	188
CE–ED	50 mM borate buffer (pH 8.7)	*Rhododendron dauricum*	farrerol, quercetin	189
CE–UV_{270}	30 mM sodium borate (pH 9.00) with 40% (v/v) MeOH	propolis	asebotin, two kaempferol glycosides, 7-methoxykaempferol glycoside, two quercetin glycosides	116
CE–UV_{214}	75 mM borate buffer, (pH 9.4)	*Panax ginseng*	phlorin	190
Chiral MEKC–UV_{210}	25 mM sodium borate (pH 10.0)–MeOH containing hydroxypropyl-β-cyclodextrin (2 mM) and hydroxylpropyl-γ-cyclodextrin (20 mM) (10:90, v/v)	flavonoids isolated from legumes by LC	vestitone	191
CE–UV_{280}	(1) MeCN–MeOH–AcOH (71.25:4, v/v) with 90 mM $AcONH_4$ (non-aq. CE) (2) 50 mM K_2SO_4 and 600 mM boric acid titrated to pH 7.0 (CZE)	black tea	three tea flavins	192
CE–ED	70 mM sodium borate (pH 9.2)	*Rhododendron dauricum*	farrerol, scopoletin, umbeliferone, hyperoside, kaempferol, quercetin	193

Abbreviations: MeCN: acetonitrile, MeOH: methanol, AcOH: acetic acid.

The practical usefulness of CE for flavonoid analysis can be illustrated by discussing a recent paper on the performance of MEKC and CZE in some detail [122]. With thirteen flavonoids as model compounds, emphasis was given to the influence of separation conditions and molecular structure on the electrophoretic behaviour of the flavonoids. The separation mechanisms of these two CE modes are fundamentally different. CZE is only applicable to charged analytes and the charge-to-size ratios determine the electrophoretic migration times. In MEKC one should distinguish between neutral and charged analytes. With the former group, separation is based on hydrophobicity, which affects the analyte partitioning between the aqueous (moving with the electro-osmotic flow) and the micellar phases (charged and migrating with a different velocity). For ionic analytes separation in MEKC is based on both the degree of ionization and the hydrophobicity [123]. The flavonoids that were studied – flavonols, flavanones, flavanonols and a flavone – all have at least one phenolic hydroxy group; ionization of this group primarily determines the electrophoretic mobility. The

magnitude of the net negative charge on a particular flavonoid is determined by the position and number of these groups and by the pH of the buffer solution. In CZE, two buffers were tested and the pH was varied over a wide range, but no conditions could be found that enabled separation of all thirteen flavonoids. With MEKC better results were obtained. The composition of the four-component MEKC buffer and the pH range were varied. The migration times of all flavonoids except the very hydrophilic anthocyanins, increased with increasing pH; optimum resolution was at pH 7.3. Apparently, the separation selectivity of MEKC is better since more molecular-structure parameters play a role than in CZE. These include the degree of saturation and the stereochemistry of the C-ring, alkyl substitution and the number and position of phenolic hydroxy groups, methylation and glycosylation of the hydroxy groups and the complexation of flavonoids with borate buffer [122]. To the best of our knowledge, this is the first study in which MEKC and CZE are compared for flavonoid analysis and in which the better resolution of the former mode is demonstrated. Unfortunately, the authors did not study any real-life samples.

Table 9. Comparison of MEKC and LC analysis of flavonoids in five root extracts [112].

	UV_{287} peak areas relative to FGM of:		
Technique	MGM	FG	MG
White sweet clover			
CE	0.35	0.29	0.09
LC	0.33	0.08	0.07
Barrel medic			
CE	0.31	n.d.	n.d.
LC	0.37	n.d.	n.d.
Fenugreek			
CE	8.85	0.30	0.15
LC	9.46	0.23	0.52
Apollo alfalfa			
CE	0.22	0.15	0.01
LC	0.16	0.04	0.03
Cimarron alfalfa			
CE	0.15	0.15	0.09
LC	0.19	0.08	0.04

Abbreviations: FG, formononetin-7-*O*-glucoside; FGM, formononetin-7-*O*-glucoside-6″-*O*-malonate; MG, medicarpin-7-*O*-glucoside; MGM, medicarpin-7-*O*-glucoside-6″-*O*-malonate; n.d., not detected. For CE conditions, see text; LC: acetonitrile–1% aq. phosphoric acid.

As regards real-life applications, Baggett *et al.* [112] used MEKC for the profiling of isoflavonoids in legume root extracts and compared it to LC. The optimum MEKC electrolyte was a mixture of 25 mM boric acid, 60 mM SDS and 1.6% 1,2-hexanediol, pH 9. Sample-to-sample and run-to-run repeatabilities for MEKC were very good if the capillary was cleaned between each injection. Most MEKC results correlated rather well with those obtained for LC (Table 9), while the runs were about two-fold faster [112]. A disadvantage was that optimization of the MEKC separation was more critical than that of LC.

In another study, CZE and LC were compared for the determination of the aglycones genistein and daidzein and their glucosides and glucoside–acetates in food products. LC was found to provide some ten-fold better UV detectability (LODs, 0.01–0.03 mg/l) and was less dependent on matrix effects [113]. In addition, in CZE resolution and repeatability were poor. The advantage of five-fold shorter run times in CZE is, of course, completely off-set by these drawbacks. CZE was also combined with SPE to determine flavone and flavonol glucosides and aglycones in *Flos lonicerae* [123]. Concentrations were 15–660 μg/g, with recoveries of 94–104% and LODs, 0.4–0.6 mg/l.

Capillary electrochromatography (CEC) has also been used for flavonoid analysis. CEC was compared with LC for the analysis of hop acids and prenylated hop flavonoids [124], and of polymethoxylated flavones in essential oils [125]. In both studies the capillary CEC column was packed with C18-bonded silica, and acetonitrile–TRIS buffer (10 mM, pH 7.8) was used for separation with UV detection. The hop extract was subjected to off-line SPE and analyzed at 30 kV and 30°C. Ten hop acids and flavonoids were identified on the basis of their UV spectra and retention times; these included two pairs of hop acid isomers. LC on a C18-bonded phase, and with acetonitrile–formic acid gradient elution gave the same elution order of all target analytes, but one pair of isomers was not fully separated.

In the study on polymethoxyflavones [125], five of these compounds present in mandarin oil were well separated by both CEC and LC; with CEC the retention times were slightly shorter and, somewhat surprisingly, in this study the retention order was the opposite of that found in LC. The samples were also analyzed with CEC in the normal-phase mode using an acetonitrile–isopropanol–hexane eluent. The retention orders in NP-CEC and RP-CEC were very similar – and so were the run times – but the resolution was better in the latter mode. Unfortunately the authors did not provide analytical performance data, which precludes a more detailed comparison of the CEC and LC methods.

Only a few papers discuss the use of CE–MS for the determination of flavonoids [117] and phenolic compounds [126]. This may indicate that the technique is not considered sufficiently robust and user-friendly by many researchers. In the CE–ESI(–)-MS study by Lafont *et al.* [126], a standard mixture of eight phenolic compounds was analyzed. Admittedly, phenolic acids are no flavonoids, but since they are very similar compounds, they will be included in the present discussion. With SIM-MS the authors were able to identify all eight compounds (*cf.* Fig. 11) based on their retention times and characteristic fragment ions ($[M–H]^-$, loss of CO, CO_2 and CH_3) and obtained LODs of 0.1–40 μg/l. Eight traces are for the analytes and two for the internal standard, *p*-chlorophenol, which has two isotopic $[M–H]^-$ ions *(m/z* 127 and 129). The authors state that it is easier than in LC to couple CE to an ESI interface since, because of the very low (nl/min) flow rate, no flow splitting is needed.

However, for successful coupling of CE to MS, the ESI interface design plays an important role; the needle should be grounded and a voltage applied to the counter electrode. In a more recent paper [117], CE–ESI(–)-MS was used for the determination of flavonoids in a phytomedicine and compared with CE–UV. MS detection sensitivity for hesperetin and naringenin (LODs, 0.5 mg/l in SIM) was similar to that of UV_{254}, while for biochanin A the LOD was less satisfactory, *i.e.* 2 mg/l. On the other hand, CE–UV was not sufficiently selective to enable quantitation, and CE–MS in the SIM mode had to be used to determine the concentration of naringenin in a liquid herbal drug at 6 mg/l. Surprisingly, in the CE–MS study quoted earlier [126], the LODs were one to two orders of magnitude lower. We do not have an explanation, since the authors did not compare their results with the former study.

At the present time, the future of CE for flavonoid analysis is, to our opinion, not too bright. Compared with LC, there is no dramatic difference of run times, and the limited consumption of sample and solvents (not particularly expensive in LC anyway) do not appear to have much impact. Moreover, the repeatability of retention/migration times still is better in LC than in CE. Probably, the most promising aspects to explore are the complementarity of CEC and MEKC separations to LC, and their robust interfacing with MS.

1.3.2.4 Thin-layer chromatography

Since the early 1960s, TLC has been used in flavonoid analysis. TLC is especially useful for the rapid screening of plant or medicinal extracts for pharmacologically active substances prior to detailed analysis by instrumental techniques such as LC–UV – especially because many samples can be analyzed simultaneously. In most cases silica is used as stationary phase, and plates are developed with either a combination of 2-(diphenyl boryoxo)ethylamine and polyethylene glycol or with $AlCl_3$. Detection is mainly performed using UV light at 350–365 or 250–260 nm or with densitometry at the same wavelengths. At present, TLC still plays a distinct role in flavonoid analysis: a representative selection of the about eighty papers published in the last five years is summarized in Table 10. Some of these are discussed below to illustrate the state-of-the-art.

One interesting example is the separation of flavonoid glycosides and rosmarinic acid from *Mentha piperita* (peppermint) on HPTLC plates [127]. A variety of (modified) silica sorbents were tested as well as many organic eluents which ranged from *n*-hexane to esters, ethers and methanol.

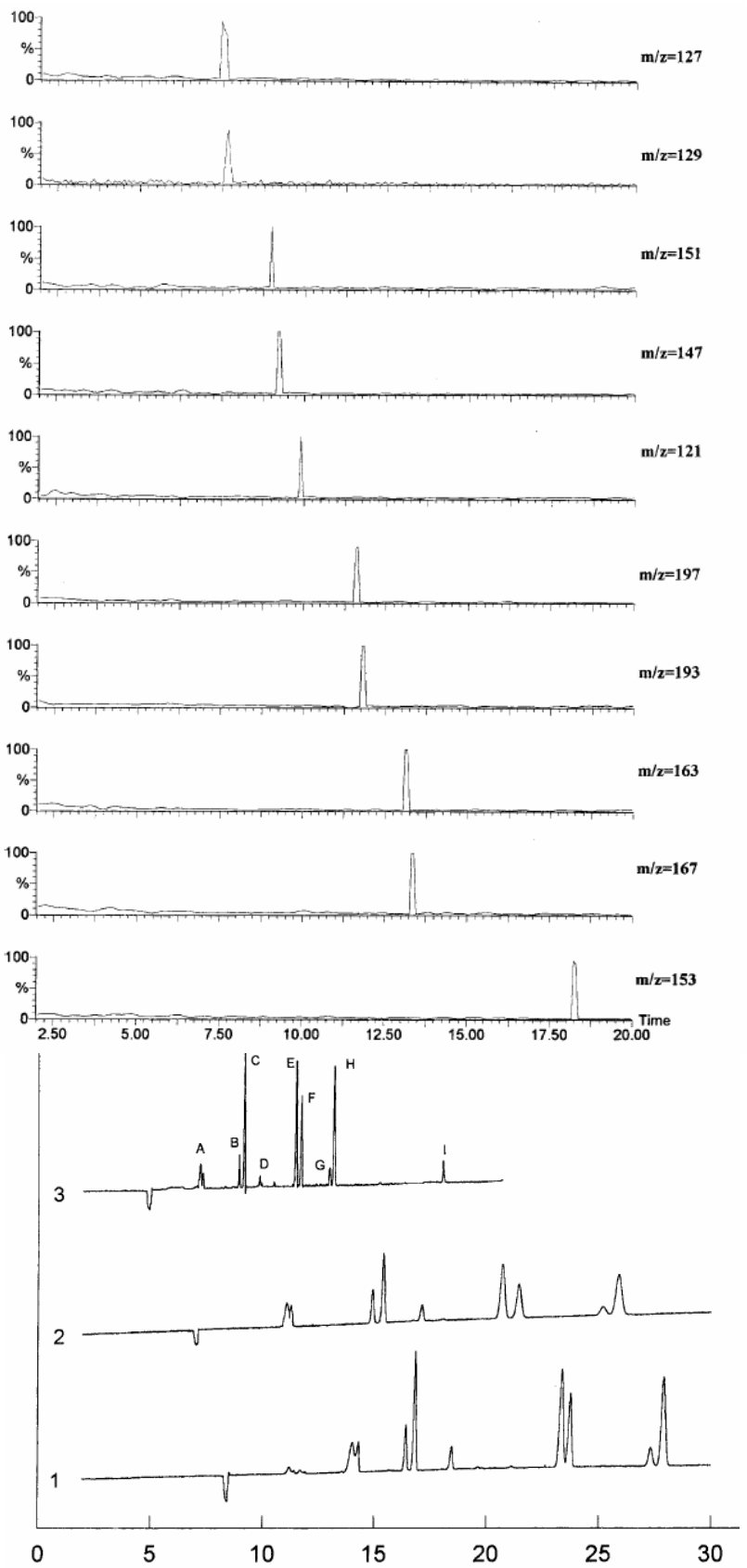


Figure 11. SIM-mode CE–ESI(–)-MS and CE–UV of a mixture of *p*-chlorophenol [I.S., two top traces] (A), vanillin (B), *trans*-cinnamic acid (C), 4-hydroxybenzaldehyde (D), syringic acid (E), ferulic acid (F), *p*-coumaric acid (G), vanillic acid (H) and protocatechuic acid (I) (each at 30 mg/l; voltage 30 kV). CE–UV with run voltages of 20 kV (1), 25 kV (2) and 30 kV (3) [126].

For six standard compounds, the best separation was obtained on aminopropyl-bonded silica with acetone–acetic acid (85:15, v/v) as eluent; with C18-bonded silica and water–methanol (60:40, v/v) good results were also achieved. The six standard compounds were found in the peppermint extract, isolated by preparative LC and their structures determined by several spectroscopic identification techniques. Eriocitrin was found to be the main flavonoid constituent; no quantification data were provided.

Soczewinski *et al.* [128] used double-development TLC to separate a flavonoid mixture containing nine glucosides and seven aglycones. In the first step, the more polar glycosides were separated using an eluent with high solvent strength. After solvent evaporation, the aglycones were separated in a second step in the same direction with another, relatively weak, eluent.

In several recent papers [129–132], numerical taxonomy is used to calculate the orthogonality of the retention factors of mixtures of flavonoids. On the basis of results calculated for nineteen standard compounds and using two parameters, the optimum eluent composition to study flavonoids in red wine [132] and in propolis [131] was determined for eleven tertiary eluents. The success of the approach was demonstrated by the fact that, for the optimal eluent combination, up to ten of the nineteen standard compounds were identified in fourteen propolis samples of different origin. Application to red wine was also successful: several phenolic compounds, including three flavonoids, were identified [132].

Quantification generally is not a main goal of TLC studies. However, densitometry is used in several studies [133, 134]. In one paper [133], kaempferol and quercetin were determined in an extract of *Ginko biloba* leaves by scanning the HPTLC silica plates in the reflectance mode at 254 nm. Recoveries using standard addition were above 94%. The concentrations of kaempferol and quercetin in the extract were 7 and 14 mg/l, respectively. Janeczko *et al.* [134] used a similar method to determine genistin and daidzin at 260 nm in various soy cultivars. The analytical performance data were fully satisfactory, possibly because the analyte concentrations were fairly high: genistin, 0.06–0.15%; and daidzin, 0.03–0.01%. In propolis, several flavonols, flavanones and phenolic acids were quantified using two-dimensional TLC with densitometry at 254 and 366 nm [135]. Also here, good analytical performance data were obtained; concentrations were 90–1440 mg/l.

Wojciak-Kosior *et al.* [136] used TLC combined with densitometry to study the hydrolysis of six flavonoid glycosides. The flavonoids were heated under reflux with HCl and analyzed every 15 min. The pseudo first-order hydrolysis rate constants varied between 1.7×10^{-2} and 1.1×10^{-2} min^{-1} for 3- and 7-glycosides; for 7-glycosides the hydrolysis was practically complete after 90–105 min. The hydrolysis mechanism of rutin, a diglycoside, was found to be more complicated. There are presumably two steps – a mechanism to be studied in the near future.

Table 10. Representative studies on TLC of flavonoids

Method	Stat. phase	Eluents (v/v)	Detection	Sample	Flavonoids	Ref.
HPTLC	silica	EtAc–FA–H_2O (82:9:9)	densitometry at 300 nm	*Passiflora* leaves	orientin, isoorientin	184
HPTLC	silica, aminopropyl-, cyanopropyl- and C18-bonded silica	various eluents were tested; Me_2O–AcOH 85:15 was the best	UV at 366 nm and (after isolation) IR	*Mentha piperita*	5 flavonoids	127
Double develop-ment	silica	EtAc–FA–H_2O (85:15:0.5) and DCM–EtAc–FA (85:15:0.5)	densitometry at 254 nm	standard mixture	9 glycosides, 7 aglycones	128
TLC	silica	toluene–EtAc–FA –MeOH (3:3:0.8:0.2)	densitometry at 355 nm	*Bacopa-monniera, Cuminum cyminum* fruit, *Achillea mille-folium* flower	luteolin	194
2D	cyanopropyl-bonded silica	Me_2O–iPrOH (6:4) and 50% MeOH or THF or 1,4, dioxane	UV at 366 nm	*Sambucus nigra*	8 flavonoids	137
2D	cyanopropyl-bonded silica	several NP and RP systems	UV at 254 and 365 nm	standard mixture	9 flavonoids	195
Numerical taxonomy	silica	EtAc–MeOH– H_2O (75:15:0), EtAc–FA– H_2O (80:10:10), EtAc –FA– AcOH–H_2O (100:11:11:27)	densitometry at 254 and 366 nm	propolis	flavonols, flavanones	135
Numerical taxonomy	silica	EtAc–FA–H_2O (65:15:20)	UV at 366 nm	*Helleborus atrorubens*	quercetin, kaempferol	130
TLC	silica	EtAc–FA– AcOH –H_2O (100:11:11:26) EtAc–FA–H_2O (8:1:1)	UV at 365 nm	5 *Hypericum* taxa plants	10 flavonoids	196
TLC	silica		densitometry at 254	standard mixture	6 flavonoid glycosides	136
Numerical taxonomy	silica	chloroform–MeOH –FA (various v/v) *n*-hexane–EtAc –AcOH (31:14:5)	UV at 366 nm	propolis		131
HPTLC	silica	EtAc–MeOH–FA (various v/v)	UV at 254 nm	standard mixture	8 aglycons, 15 glycosides	197

EtAc: ethyl acetate, FA: formic acid, AcOH: acetic acid, MeOH: methanol, DCM: dichloromethane, iPROH: isopropanol.

2D-TLC on cyanopropyl-bonded silica was used to separate eight flavonoids and three phenolic acids in *Flos sambuci* L. [137]. The first dimension was a normal-phase separation for which seven binary eluents were tested, and the second one a reversed-phase separation, studied by using three binary eluents. From amongst the 21 combinations, the three best ones all contained *n*-hexane in the first, and water in the second dimension. Fig. 12 shows the results of a separation using the eluents 40% propan-2-ol in *n*-hexane in the first dimension and 50% aqueous 1,4-dioxane in the second dimension, although errors in migration direction in their graphs and compound numbering complicate the interpretation of their data. More than twelve spots can be discerned and nine flavonoids and three phenolic acids were (tentatively) identified in the *Flos sambuci* extract.

In summary, several modes of TLC are still in vogue for flavonoid analysis. The emphasis is on screening for the main flavonoids in real-life samples. In most cases a close-to-standard protocol can be followed, but a newer method such as numerical taxonomy also deserves attention.

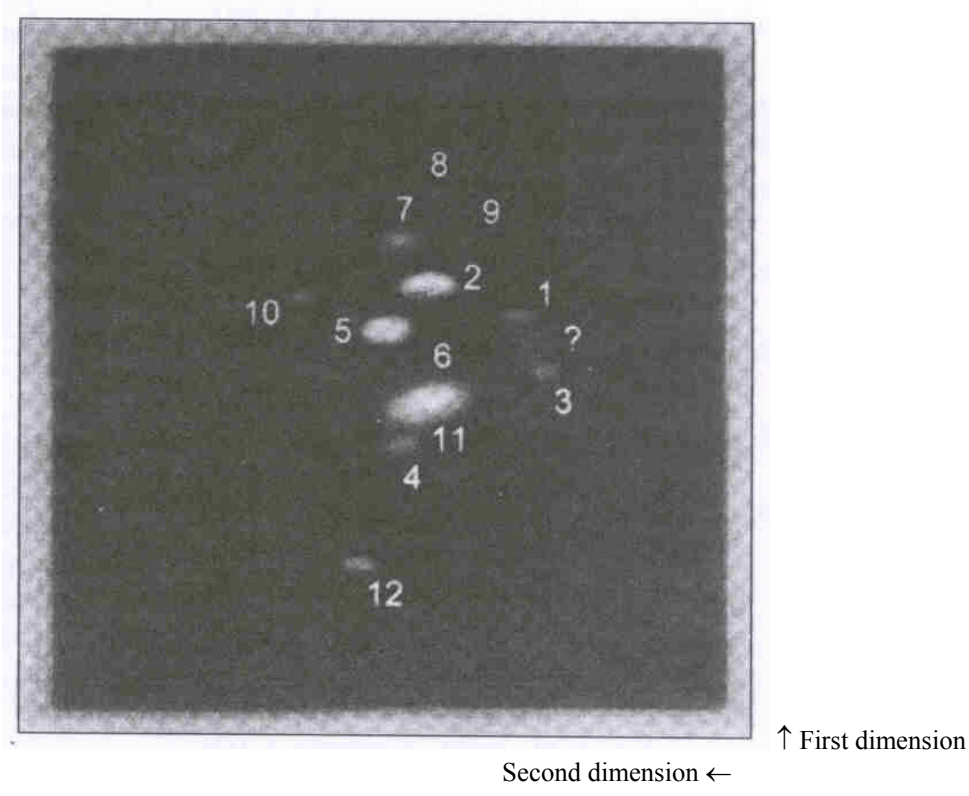


Fig. 12. 2D-TLC of *Flos sambuci* extract on cyanopropyl-bonded silica. Spot assignment: 1, myricetin; 2, naringenin; 3, luteolin; 4, apigenin; 5, acacetin; 6, hyperoside; 7, quercetin; 8, naringin; 9, rutin; 10, hesperetin; 11, quercitrin; 12, astragalin. Eluents: first dimension, 40% propan-2-ol in *n*-hexane; second dimension, 50% aqueous 1,4-dioxane. UV detection at 366 nm after development with 2-(diphenyl boryoxo)ethylamine and polyethylene glycol [137].

1.4 Identification and structural characterization

1.4.1 General

Today, LC–MS/MS is the most important technique for the identification of target flavonoids and the structural characterization of unknown members of this class of compounds. As regards target analysis, tandem-MS detection has largely replaced single-stage MS operation because of the much better selectivity and the wider-ranging information that can be obtained. Depending on the nature of the application, additional information is derived from LC retention behaviour, and UV absorbance – and, occasionally, FLU or ED – characteristics, due comparison being made with standard injections and/or tabulated reference data. In studies on the characterization of unknowns, a wide variety of LC–MS/MS techniques is usually applied next to LC–DAD UV for rapid class identification. In addition, LC–NMR often turns out to be an indispensable tool to arrive at an unambiguous structural characterization.

In Section 4.2, attention will be devoted to the main fragmentation pathways for four major classes of flavonoids, *i.e.*, flavones, isoflavones, flavonols and flavanones. In this context, the retro-Diels–Alder (RDA) reaction, which is an important fragmentation reaction of flavonoids, will be discussed. RDA fragments are especially important for the structural characterization of aglycones and the aglycone part of flavonoid conjugates. Next, in Section 4.3, the on-line coupling of LC and NMR will be discussed, and its increasing importance for the characterization of flavonoid conjugates illustrated. This part of the text will also serve to demonstrate the complementary roles of, specifically, NMR- and MS/MS-based information. Off-line NMR, which has been used extensively for flavonoid analysis, is outside the scope of this review and will not be considered.

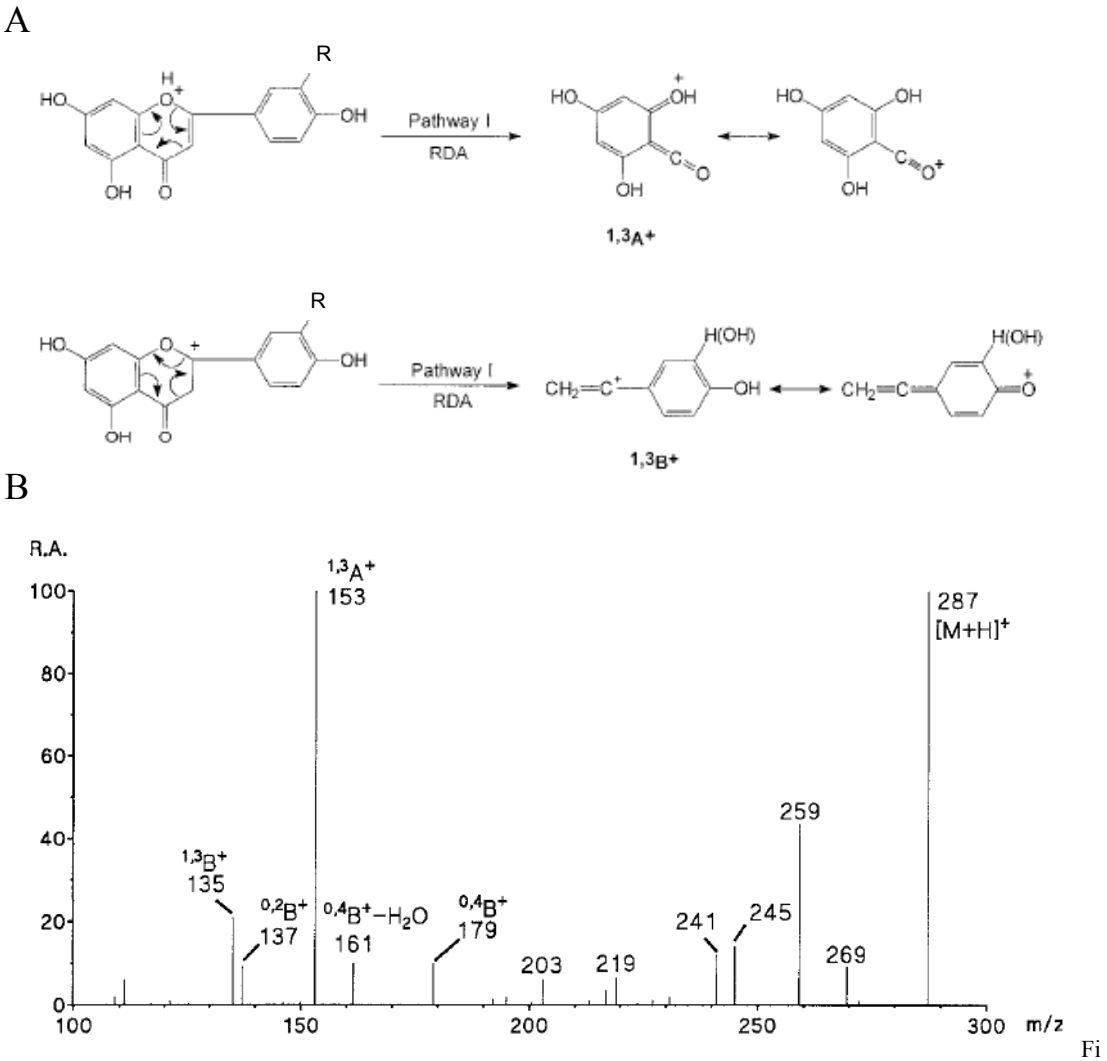


Fig. 13. (A) RDA reaction mechanisms for the formation of $^{1,3}A^{+}$ and $^{1,3}B^{+}$ fragment ions for apigenin (R=H) and luteolin (R=OH). Each arrow represents relocation of one pair of electrons. (B) Low-energy MS/MS spectrum of luteolin [93].

1.4.2 LC–MS/MS

1.4.2.1 General

In order to facilitate discussions on the mass fragmentations of flavonoid aglycones, Ma *et al.* [138] proposed a nomenclature to unambiguously describe the resulting fragment ions (Fig. 13). In the PI mode, the ions that are formed after the cleavage of two bonds in the C-ring, are denoted $^{i,j}A^{+}$ and $^{i,j}B^{+}$, with ion A containing the A-ring and ion B, the B-ring. The indices i and j represent the C-ring bonds which are broken. When the NI mode is used, the ions are denoted $^{i,j}A^{-}$ and $^{i,j}B^{-}$, respectively. Ions derived from the fragment ions by the loss of a fragment X, are denoted $[^{i,j}A^{\pm}–X]$ and $[^{i,j}B^{\pm}–X]$, respectively.

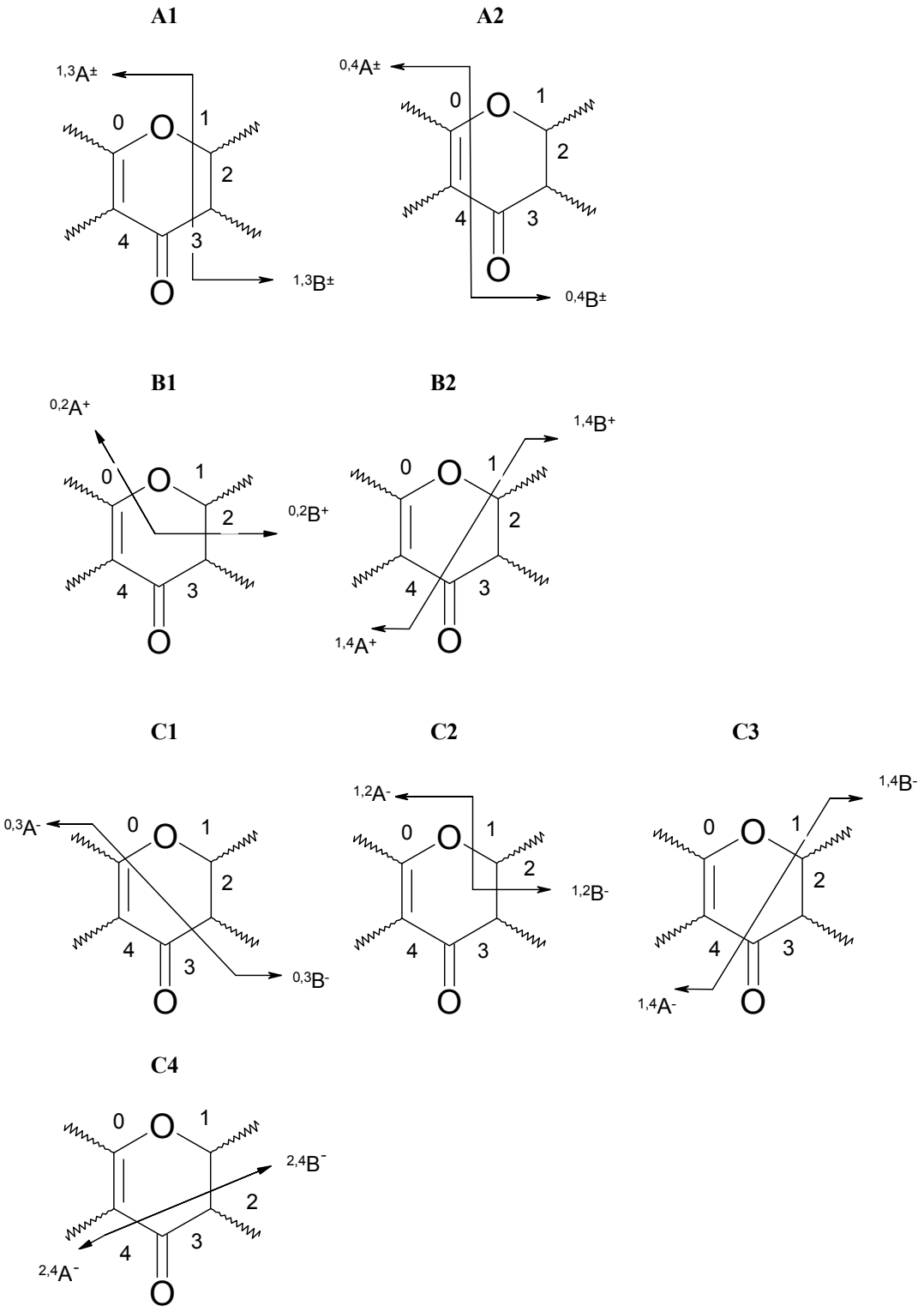


Fig. 14. Fragmentation pathways for flavonoids caused by cleavage of C-ring bonds; A, in both PI and NI: (A1) 1 and 3, (A2) 0 and 4; B, in PI: (B1) 0 and 2, (B2) 1 and 4; C, in NI: (C1) 0 and 3, (C2) 1 and 2, (C3) 1 and 4, (C4) 2 and 4.

As mentioned above, an important fragmentation reaction of flavonoids is the RDA reaction which may occur in six-membered cyclic structures containing a double bond and involves the relocation of three pairs of electrons in the cyclic ring. The net result of these rearrangements is the cleavage of two σ bonds and the formation of two π bonds – for

example, cyclohexene will fragment into butadiene and ethylene. Two (complementary) fragments, $^{1,3}A^+$ and $^{1,3}B^+$, are formed and charge retention can occur on either side of the cleavages, as depicted in Fig. 13A for the flavones apigenin and luteolin [138]. The C-ring cleavage product ions can be used to determine the number and nature of the substituents on the A- and B-rings. For example, in the MS/MS spectra of apigenin and luteolin (the latter is shown in Fig. 13B), which have $[M+H]^+$ *m/z* 271 and 287, respectively, a $^{1,3}A^+$ fragment ion shows up at *m/z* 153. The corresponding $^{1,3}B^+$ ions are found at *m/z* 119 and 135, respectively. This indicates that the two compounds differ in the substitution of the B-ring, with luteolin having two –OH groups, and apigenin only one [138].

When reading the more detailed discussions on MS fragmentation behaviour presented below, one should consider that, in the quoted studies, different instruments and operating conditions were often used. Fortunately, experience shows that the fragmentation pathways are largely independent of the ionization mode (ESI or APCI) and the type of instrument (triple quadrupole or ion trap) used [84, 4]. On the other hand, significant differences do occur as regards the relative abundances of the various fragment ions. These are, therefore, not included in the discussions presented in Sections 4.2.2–4.2.3 below.

1.4.2.2 Fragmentation in PI mode

Relevant information on the main fragment ions formed after cleavage of the C-ring of the selected flavonoid classes in the PI mode (*cf.* Fig. 14), is summarized in Table 11.

RDA cleavage which generates $^{1,3}A^+$ and $^{1,3}B^+$ fragment ions (Fig. 14, A1), is the most important fragmentation pathway for flavanones, flavones and flavonols, but also occurs with isoflavones. The first three classes all show $^{1,3}A^+$ as the most prominent product ion, with the flavones luteolin and apigenin, the flavonol kaempferol [138], and the flavanones naringenin and hesperetin [98] as typical examples. The $^{1,3}B^+$ ion is also observed, and was even proposed as a diagnostic ion for flavones [139]; however, it is also formed with flavones and a flavanone such as naringenin. Compounds having a methoxy substituent show relatively weaker RDA fragmentation [138]: in the spectra of acacetin and chrysoeriol, the $^{1,3}A^+$ ion typically has a less than 10% relative abundance [138]. The $^{1,3}A^+$ fragment ion was also reported for isoxanthohumol – a chalcone with 6-prenyl substitution – and 6- and 8-prenylnaringenin, although with less than 20% relative abundance [140]. If a low collision energy of 30 V was used, the mass spectra of prenylated flavones and flavonols showed $[^{1,3}A–C_4H_8]^+$ as the only product ion. In a study on the flavonoids of *Humulus lupulus*, this characteristic loss of the isoprenyl substituent has been used for the target analysis of prenylflavonoids in an SRM procedure [141].

Table 11. Fragment ions observed for the selected flavonoid classes in the PI mode.

Compound	Substituents		Fragment ions											Ref.
	–OH	$–OCH_3$	$[M+H]^+$	$^{0,2}A^+$	$^{0,2}B^+$	$^{1,3}A^+$	$^{1,3}B^+$	$^{1,4}A^+$	$^{0,4}B^+$	$[M+H–15]^{\cdot+}$ ($–CH_3^{\cdot}$)	$[M+H–56]^+$ (–CO–CO)	$[M+H–42]^+$ ($–C_2H_2O$)	$[M+H–46]^+$ ($–H_2O–CO$)	
Flavones														
Apigenin	5,7,4’		271	-	121	153	119	-	163	-	-	229	225	93
Luteolin	5,7,3’, 4’		287	-	137	153	135	-	179	-	-	245	241	93
Acacetin	5,7	4’	285	-	135	153	133	-	177	270	-	243	239	93
Chrysoeriol	5,7,4’	3’	301	-	151	153	149	-	-	286	-	259	-	93
Flavonols														
Kaempferol	5,7,4’		287	165	121	153	-	-	-	-	231	245	213	93
Quercetin	5,7,3’,4’		303	165	137	153	-	-	-	-	247	262	229	93
Myricetin	5,7,3’,4’,5’		319	165	153	153	-	-	-	-	263	277	245	93
Isorhamnetin	5,7,4’	3’	317	165	151	153	-	-	-	302	261	275	-	93
Galangin	5,7		271	165	-	153	-	-	-	-	215	229	225	140
Flavanones														
Naringenin	5, 7, 4’		273	-	-	153	119	147	-	-	-	231	227	139
Isoxantho-humol	7,4’	5	355	-	-	235	-	-	-	-	-	-	-	141
8-Prenyl-naringenin	5,7,4’		341	-	-	221	-	-	-	-	-	-	-	141
6-Prenyl-naringenin	5,7,4’		341	-	-	221	-	-	-	-	-	-	-	141
Isoflavones								-						
Daidzein	7,4’		255	-	-	137	119[1]	-	-	-	199	-	-	84
Genistein	5,7,4’		271	-	-	153	119	-	-	-	215	-	-	84
Formono-netin	7	4’	269	-	-	137	133	-	-	254	213	-	-	84
Biochanin A	5,7	4’	285	-	-	153[1]	133	-	-	270	229	-	-	84,119

[1] Not detected in [84].

For flavones and flavonols cleavage of the 0,2 bonds (Fig. 14, B1) is a common C-ring cleavage pathway. Various authors have reported the formation of the $^{0,2}B^+$ ion with relative abundances ranging from 1 to 90%, *e.g.* for kaempferol, quercetin, myricetin, isorhamnetin, apigenin, luteolin, acacetin and chrysoeriol [25, 138]. The corresponding $^{0,2}A^+$ ion may be used to distinguish flavonols since it does not occur in the spectra of other classes of flavonoids [138]. Interestingly, ions due to the 0,2 cleavage reaction are not reported in the NI mode.

The 0,4 C-ring cleavage (Fig. 14, A2) is not often discussed in the literature. Only protonated flavones fragment via this pathway, *viz.* under low-energy FAB-CID conditions [138]. Consequently, the presence of $^{0,4}B^+$ ions can be considered diagnostic for flavone aglycones. To the best of our knowledge, the cleavage of bonds 1 and 4 of the C-ring (Fig. 14, B2) has been reported only once, *viz.* to explain the *m/z* 147 ion ($^{1,4}A^+$) in the spectrum of naringenin [139]. However, one should add that the *m/z* 147 fragment may also correspond to a $^{0,4}B^+$ ion after loss of water.

Other, generally less characteristic, fragments common to most flavonoids are those arising from the loss of H_2O (18 Da), CO (28 Da), C_2H_2O (42 Da) and the successive loss of H_2O and CO (46 Da). However, to the best of our knowledge (see Table 11), the fragments $[M+H-42]^+$ ($-C_2H_2O$) and $[M+H-46]^+$ (successive loss of H_2O and CO), do not show up in the spectra of isoflavones. A loss of 68 Da (successive loss of C_2H_2O and C_2H_2), which involves a more complex fragmentation process, has also been observed, and has been suggested to be indicative for flavones [140]. However, there are exceptions to this rule [138]. Similarly, the presence of $[M+H-56]^+$ (loss of 2 × CO) has been suggested as an 'indicator' for isoflavones [84, 92, 140). However, the same fragment has also been observed for several flavonols [138, 139, 142].

As can be seen from Table 11, the product ion $[M+H-15]^{+\bullet}$, formed by the loss of a methyl radical, is prominent in many *O*-methylated isoflavones, flavones and flavonols [*e.g.* 25, 84, 92 and 143]. In addition to the loss of a methyl radical, further loss of water or H_2O-plus-CO may occur, resulting in $[M+H-33]^{+\bullet}$ and $[M+H-61]^{+\bullet}$ fragments. In the mass spectra of 3-methoxyflavonoids, $[M+H-15]^{+\bullet}$ was found to be accompanied by an $[M+H-16]^+$ ion of equal or somewhat lower abundance. For this ion, the loss of CH_4 was proposed by the formation of a furan ring involving $C_{2'}$ and the oxygen at C_3 [25].

1.4.2.3 Fragmentation in NI mode

Relevant information on fragment ions of the selected flavonoids observed in the NI mode, which has been studied more frequently than PI, is summarized in Table 12.

The RDA C-ring cleavage of the 1,3 bonds, which creates $^{1,3}A^-$ and $^{1,3}B^-$ product ions (Fig. 14, A1), is the most important fragmentation pathway in the NI mode, as is also true for the PI mode. As Table 12 shows, $^{1,3}A^-$ and $^{1,3}B^-$ fragments are reported for many flavonoids. In the mass spectra of the flavonols kaempferid, eriodictyol, morin, quercetin and rhamnetin [144], and the prenylated flavonoids, 8- and 6-prenylnaringenin [141] $^{1,3}A^-$ were the most abundant fragment ions with $^{1,3}B^-$ in second place. In another study [50], however, the relative abundances of the $^{1,3}A^-$ and $^{1,3}B^-$ ions of luteolin and genkwanin were found to be quite low (1-10%), and in a third one, kaempferol did not show any RDA ions at all [144]. These mutual differences possibly reflect differences in the experimental set-up and/or operating conditions. As for kaempferol, it has been reported that this compound shows little fragmentation up to a collision energy of about 25 eV [139]. However, in the study quoted above no fragmentation was observed even at a collision energy of 30 eV. All of this shows that one has to be careful when attributing an 'indicator role' to specific fragments.

Another C-ring cleavage generates $^{0,3}A^-$ and/or $^{0,3}B^-$ fragments (Fig. 14, C1). In a study of 14 isoflavones, flavones and flavanones, $^{0,3}B^-$ fragments were observed only for the isoflavones daidzein and genistein; however, the same fragment was also observed for a flavone [139]. Based on the presence of *m/z* 135 and 148 ions, which, according to them, represented $^{0,3}A^-$ and $[^{0,3}B–CH_3^{\bullet}]^-$ fragments, respectively, Prasain *et al.* [140] tentatively identified an unknown compound in a kudzu dietary supplement extract as 3'-methoxydaidzein. The authors referred to an earlier study of methoxylated flavones [145] in which similar fragments were observed, but did not provide additional (NMR) data to prove that the methoxy group is in the 3' position.

$^{0,4}A^-$ and $^{0,4}B^-$ fragments (Fig. 14, A2) are observed at relatively low abundance for at least some members of all classes of flavonoids discussed here (Table 12). A $^{0,4}A^-$ fragment was observed for kaempferol [139], for several flavonoids [144] and for the flavone apigenin, the flavonols quercetin and kaempferid, and the flavonones eriodictyol, naringenin and isosakuremetin [50]. In a study of several isoflavones and flavones, fragmentation of isoflavones mainly resulted in $^{0,4}B^-$ ions, whereas for the flavones $^{1,3}A^-$ was more prominent (84) (*cf.* Table 12). 0,4 C-ring cleavage was also proposed for a fragment ion of the flavanone isosakurametin [50]: loss of $–CH_3^{\bullet}$ from the deprotonated molecule was followed by a 0,4 C-ring cleavage to create $[^{0,4}B–CH_3^{\bullet}]^-$.

1,2 C-ring cleavage (Fig. 14, C2) was suggested for the main ions ($^{1,2}A^-$) of the flavonols quercetin (*m/z* 179) and fisetin (*m/z* 163) [50]. The complementary ions ($^{1,2}B^-$, *m/z*

121 for both compounds) were also observed, but with much lower abundances. For quercetin, the same fragment ions were also reported in two other studies [139, 144], although they were not designated as $^{1,2}A^-$ and $^{1,2}B^-$ there. Because other flavonols that were studied did not show 1,2 C-ring cleavage, the authors proposed that this pathway is specific for 3',4'-dihydroxyflavonols [50]. It is interesting to add that, in another paper [144], *m/z* 121 in the spectrum of the 3',4'-dihydroxyflavonol rhamnetin was attributed to $^{0,4}A^-$ rather than $^{1,2}B^-$. $^{1,2}A^-$, but no $^{1,2}B^-$, ions have also been observed in the spectra of two isoflavones, formononetin and biochanin A [84, 119].

The cleavage of bonds 1 and 4 (Fig. 14, C3) has been proposed to explain the formation of *m/z* 149, $[^{1,4}B+2H]^-$, in the mass spectrum of apigenin. The assignment was made because few other structures were considered acceptable; MS^3 experiments did not provide more information [50]. However, Hughes *et al.* proposed that the *m/z* 149 ion is either $^{0,4}B^-$ or $^{0,3}B^-$. The authors favoured the latter cleavage, because in that case a subsequent loss of 2 × CO may occur, which had earlier been observed for the $[M–H]^-$ ion of galangin (3,5,7-trihydroxyflavone) [139]. An *m/z* 149 ion has also been found in the spectrum of luteolin [144]. However, it is probably not the equivalent of the *m/z* 149 ion of apigenin: since luteolin has an additional hydroxy substituent on the B-ring, the ion would have a higher mass.

Next to the various C-ring cleavages, other fragmentations are also observed in the NI mode. To give an example, the loss of *m/z* 15 from the deprotonated molecule indicates the loss of a methyl radical, as was also observed in the PI mode. This product ion was reported for the isoflavones formononetin and biochanin A [92, 84], and also for two of their 7-*O*-glycosidic conjugates, ononin and sissotrin [84]. Hesperetin and its 7-*O*-glycoside, hesperidin, also yielded the $[M–H–15]^-$ ion. The same fragment is prominent in the spectra of methoxylated flavonoids such as acacetin, isorhamnetin, rhamnetin and hesperetin (Table 12) [144]. It is interesting to note the absence of B-ring fragments in the MS/MS spectra of acacetin (methoxylated in the B-ring), hesperetin and isorhamnetin [144].

The loss of small fragments such as CO and H_2O occurs in the NI as well as in the PI mode. For example, after cleavage of bonds 2 and 4, the deprotonated molecule loses two CO moieties but does not form $^{2,4}A^-$ and $^{2,4}B^-$ fragment ions. This fragmentation pathway was suggested to explain the *m/z* 213 ion of galangin [139]. Two further comments are: (i) as in the PI mode, $[M–H–56]^-$ only occurs for isoflavones and flavonols; (ii) the $[M–H–72]^-$ ion (successive loss of CO_2 and CO) which, to the best of our knowledge, has not been reported in PI, was observed for all selected classes except the flavanones.

Table 12. Fragment ions observed for the selected flavonoid classes in the NI mode.

Compound	Substituents		Fragment ions														Ref.
	–OH	$–OCH_3$	$[M–H]^-$	$^{0,3}A^-$	$^{0,3}B^-$	$^{0,4}A^-$	$^{0,4}B^-$	$^{1,2}A^-$	$^{1,2}B^-$	$^{1,3}A^-$	$^{1,3}B^-$	$^{1,4}A^-$	$^{1,4}B^-$	$[M–H–15]^{\cdot-}$ ($–CH_3^{\cdot}$)	$[M–H–56]^-$ (–CO–CO)	$[M–H–72]^-$ ($–CO_2–CO$)	
Flavones																	
Flavone			221	-	-	-	-	-	-	-	-	-	-	-	-	-	50
7-Hydroxyflavone	7		237	-	-	-	-	-	-	-	-	-	-	-	-	165	50
4'-Hydroxyflavone	4'		237	-	-	-	-	-	-	-	117	-	-	-	-	-	139
6,4'-Dihydroxy-flavone	6,4'		253	-	134	-	-	-	-	-	117	-	-	-	-	-	139
Genkwanin	4'	7	283	-	-	-	-	-	-	151	-	-	-	268	-	-	50
Apigenin	5,7,4'		269	-	-	107^2	-	-	-	151	117	-	149^4	-	-	-	139,50,144
Chrysin	5,7		253	-	-	-	-	-	-	151^1	101^1	-	-	-	-	181	139
Acacetin	5,7	4'	283	-	-	107^2	-	-	-	151	133	-	-	268	-	-	144
Luteolin	5,7,3',4'		285	-	-	107^2	-	-	-	151	133	-	-	-	-	213	50,144
Flavonols																	
Galangin	5,7		269	-	-	-	-	-	-	-	-	-	-	-	213	197	139
Kaempferol	5,7,4'			135^3		$107^{2,3}$	-	-	-	151^3	-	-	161^3	-			139
Kampferid	5,7	4'	299	-	-	-	-	-	-	151^5	132^5	-	-	284^5	228^5	212^5	50
Quercetin	5,7,3',4'		301	-	-	107^2	-	179	121	151	-	-	-	-	-	229	139,50
Morin	5,7,2',4'		301	-	-	107^2	-	-	-	151	-	-	-	-	-	-	144
Rhamnetin	5,3',4',	7	315	-	-	121	-	-	-	165	-	-	-	300	-	-	144
Isorhamnetin	5,7,4'	3'	315			107^2	-	-	-	151	-	-	-	300	-	-	144
Fisetin	7,3',4'		285	-	-	-	-	163	121	-	-	-	-	-	229	213	144
Flavanones																	
Naringenin	5, 7, 4'		271	-	-	107^2	-	-	-	151	119	-	-	-	-	-	139,50,144
Hesperetin	5,7,3'	4'	301	-	-	107^2	-	-	-	151	-	-	-	286	-	-	144
Eriodictyol	5,7,3',4'		287	-	-	107^2	-	-	-	151	135	125	-	-	-	-	50,144
Isosakurametin	5,7	4'	285	-	-	-	-	-	-	151	-	125	-	270	-	-	50
8-Prenylnaringenin	5,7,4'		-	-	-	-	-	-	-	219	119	-	-	-	-	-	141
6-Prenylnaringenin	5,7,4'		-	-	-	-	-	-	-	219	119	-	-	-	-	-	141
Isoflavones																	
Daidzein	7,4'-		253	-	133	-	-	-	-	-	117	-	-	-	197	181	139,112,140
Genistein	5,7,4'-		269	-	133^1	$107^{1,2}$	163	-	-	-	-	-	-	-	-	197	139,50,112
Formononetin	7	4'	269	-	-	-	177	213	-	254	-	-	-	252	-	195	84,92
Biochanin A	5,7	4'	285	-	-	107	177	229	-	270	-	-	-	268	-	211	84,92
3'-Methyldaidzein	7,4'-	3'	283	135	148	-	-		-	-	-	-	-	268	-	-	140

[1] Not detected in [50]. [2] Different fragmentation pathways proposed; see text. [3] Not observed in [143]. [4] Represents $[^{1,4}B+2H]^-$ rather than $^{1,4}B^-$ [50]. [5] From $[M–H–CH_3]^-$ rather than $[M–H]^-$ [50].

1.4.2.4 Flavonoid–(di)glycosides

Flavonoids commonly occur as flavonoid–*O*-glycosides; the 3- and 7-hydroxyl groups are the typical glycosylation sites. Glucose is the most frequently found sugar moiety, with galactose, rhamnose, xylose and arabinose in second place. Flavonoid–diglycosides are also found in nature rather frequently, with rutinose (rhamnosyl-(1→6)-glucose) and neohesperidose (rhamnosyl-(1→2)-glucose) being the most common sugar moieties. Flavonoid–*C*-glycosides – in which the sugar is directly linked to the aglycone by a C–C bond – comprise flavonoid–mono- and di-*C*-glycosides and *O*,*C*-diglycosides (with this group, the *O*-glycoside moiety is linked either to a hydroxyl group of the aglycone or to a hydroxyl group of the *C*-bound glycosyl residue). To date, *C*-glycosylation has only been found at the C-6 and C-8 positions of the flavonoid aglycone [146].

The fragment ions for glycoconjugates are denoted according to Domon and Costello [147]. Fig. 15 shows examples for naringenin–7-*O*-neohesperidose and naringenin–7-*O*–glucoside. Y represents the diglycoside, with fragments that contain the aglycone part being denoted Y_1 (loss of one glycose moiety) and Y_0 (loss of two glycose moieties); the corresponding glycose fragments are denoted B_1 and B_0, respectively. Ions formed due to cleavage in the glycose ring, and which contain the aglycone part, are labelled $^{k,l}X_j$, where j is the number of the interglycosidic bonds broken, counting from the aglycone; the superscripts k and l indicate the interglycosidic bonds, with the glycosidic bond linking the glycose part to the aglycone being numbered 0. MS/MS of flavonoid–(di)glycosides is a useful tool to differentiate (i) the 1→2 and 1→6 glycose linking types of diglycosides, and also to distinguish (ii) *O*-glycosidic (3-*O*- and 7-*O*-) and (iii) *C*-glycosidic (6-*C*- and 8-*C*-) flavonoids. Recently, an extensive tutorial on the use of mass spectrometry in the structural analysis of flavonoids was published [148]. In this overview, much attention was devoted to the characterization of the various groups of flavonoid–(di)glycosides. The present text is therefore limited to a discussion of several recent studies.

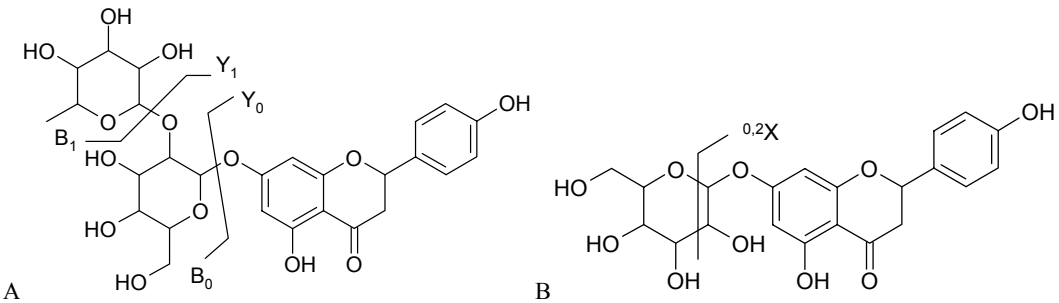


Fig. 15. Carbohydrate ion nomenclature for (A) naringenin–7-*O*-neohesperidose (naringenin–7-*O*-rhamnosyl-(1→4)-glucose) and (B) naringenin–7-*O*–glucoside [147].

In a recent paper, the interglycosidic linking types and the types of *O*-glycosidic linkage of eight flavonol, flavone and flavanone diglycosides were studied by means of LC–(+)-ESI-MS/MS [149]. As an example, in Fig. 16 the MS/MS spectra of naringenin–7-*O*-rutinoside (*O*-rhamnosyl-(1→6)-glucose) and naringenin–7-*O*-neohesperidose (*O*-rhamnosyl-(1→2)-glucose) are shown; the Y_1 ion is formed after loss of a rhamnose unit (146 Da), and the Y_0 ion after further loss of a glucose unit (162 Da). The interglycosidic linking can be determined on the basis of the value of the ratio Y_0^-/Y_1^-: when $[M+H-146]^+$ (Y_0^-) > $[M+H-(146+162)]^+$ (Y_1^-), this indicates a 1→6 linkage, while $[M+H-146]^+$ < $[M+H-(146+162)]^+$ indicates a 1→2 linkage. The type of *O*-glycosidic linkage can be determined by the presence of the Y^* ion, which corresponds to the loss of an internal glucose residue from the product ion ($[M+H-162]^+$). This ion is only observed for flavonoid–7-*O*-rutinosides and 7-*O*-neohesperidosides, and not for the corresponding 3-*O*-linked types. In another study on flavonoid–rutinosides and neohesperidosides similar conclusions were reached concerning linking types [150].

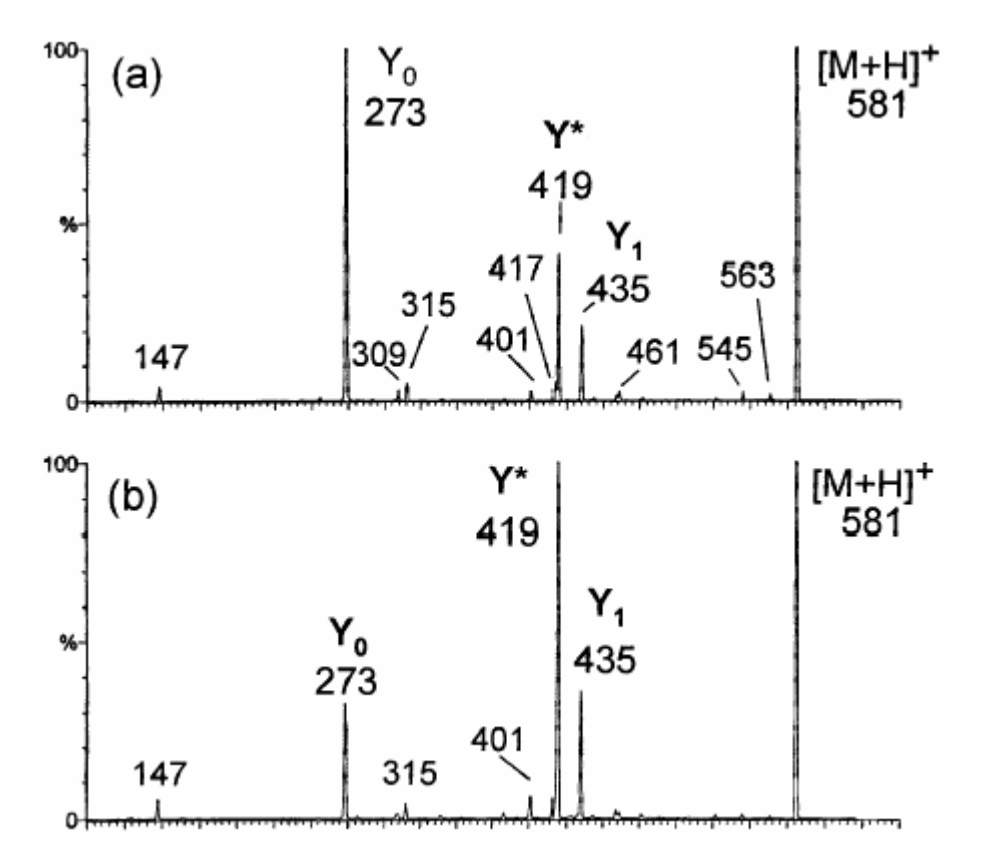


Fig. 16. MS/MS spectra obtained for $[M+H]^+$ ions of (a) naringenin–7-*O*-neohesperidoside and (b) naringenin–7-*O*-rutinoside, using LC–ESI(+)-MS/MS [149].

Recently, the above results were used to determine the glycose linking type of isoflavone–diglycosides in a *L. corniculatus* L. extract using LC–APCI(–)-MS/MS [98]. Two flavonoid–*O*-diglycosides with the same mass were found. In the mass spectra of both compounds the sequential loss of a rhamnose (Y_0^-) and glucose (Y_1^-) was observed. On the basis of the value of the ratio Y_0^-/Y_1^-, and the absence of Y^*, the two flavonoids were both identified as 3-*O*-rutinosides of kaempferol. In one of the isomers probably one or more –OH groups are shifted.

Waridel *et al*. [151] studied the MS/MS fragmentation of 6-*C*- and 8-*C*-flavonoid–glycosides. In full-scan MS, fragment ions were observed that were formed by 0,2 cleavage of the glycosidic ring (see Fig. 15B) containing the aglycone part of the flavonoid. Upon further fragmentation with MS/MS, $^{1,3}B^+$ and $^{0,2}B^+$ ions (*cf.* Section 4.2.2) were observed for both the 6-*C*- and 8-*C*-isomers. However, $^{1,3}A^+$ ions were found only for the latter isomers and could, therefore, be used as an indicator. In a kudzu extract several isoflavone–*O*- and *C*-glycosides were found using LC–ESI(+)-MS/MS [140]. $[M+H-120]^+$ was found to be the diagnostic ion for the C-glycosides. This diagnostic ion was also used to identify two xanthone–*C*-glycosides in a mango peel extract using LC–ESI(+)-MS/MS [152].

In an LC–ESI-(±)-MS/MS study of a *Crataegus* extract [146] ten flavonoid–(di)glycosides, including two acetates, were identified based on earlier information regarding the main flavonoid constituents and MS/MS results for the determination of the sugar linking type. The sugar moieties were *O*-bound glucoside, glucopyranoside, galactopyranoside and rutinoside. Loss of one or more glycose moieties was observed, *e.g.* the sequential loss of rhamnose and glucose for the rutinoside rutin, and cleavage of the interglycosidic linkage generating $^{0,2}X_1$ and $^{0,2}X_0$ fragments for vitexin–2"-O-rhamnoside, vitexin–2"-*O*-rhamnoside–acetate and isovitexin–2"-*O*-rhamnoside–acetate.

1.4.3 LC–NMR

In recent years, on-line (though often stopped-flow) LC–NMR has attracted increasing attention in the field of natural product research. The main advantages (*e.g.*, high information content, differentiation of isomers and substitution patterns) and disadvantages (low sensitivity, expensive instrumentation, long run times) have been discussed and highlighted in several recent reviews [153, 154] and there is no need to further consider them here. What should, however, be emphasized is that, while NMR detection is particularly powerful for the differentiation of isomers, sugar configurations and substitution patterns on aromatic ring systems, (tandem) MS techniques are needed to obtain information on, *e.g.,* molecular mass and functional groups. Moreover, for a comprehensive structural elucidation of a novel natural product, preparative isolation is often still necessary because in LC–NMR usually part of the ^1H spectral region is lost and, moreover, LC–NMR in most cases does not provide the

indispensable ^{13}C-NMR data [153]. The reader is referred to the same review for a discussion on the merits and demerits of hypernated techniques (LC–NMR–MS and LC–NMR–MS/MS, with or without a UV detector) compared with two separate hyphenated, LC–NMR and LC–MS, systems.

Table 13 illustrates the recent interest in LC–NMR for flavonoid analysis. In most of the cited publications, the stopped-flow mode was used to enable very long scan times to record the NMR spectra; scan times varied between one hour and several days per chromatographic peak. An alternative is to use very low flow rates as was, *e.g.*, done in a study on the flavonoid constituents of the roots of *Erythrina vogelii* where a flow rate of 0.1 ml/min was used [155]. In this study accurate mass data were acquired by means of LC–Q-TOF MS and several prenylated isoflavones and isoflavanones were identified in the root extract.

In a study of a *Gentiana ottonis* extract, LC–NMR was combined with LC–DAD UV and LC–MS/MS, and DAD UV, MS/MS and NMR spectra of the main chromatographic peaks were obtained [156]. As an example, the identification of an unknown constituent in the extract is shown in Fig. 17. The UV spectrum showed the characteristics of a flavone (*cf.* Table 4). Based on characteristic fragments observed in the MS/MS spectrum, the compound was determined to be a 6-*C* glycoside with a monohydroxylated B-ring. The NMR spectrum provided the additional information for the unequivocal identification of the flavone as swertisin. The authors claim that the lowest detection level for LC–NMR was about 0.05 μmol per peak in the on-flow mode and that in the stopped-flow mode about 100-fold less material was required (but the acquisition times then were extremely long).

The need to use several complementary techniques was also apparent in the analysis of a *Hypericum perforatum* extract. To identify its constituents a combination of stopped-flow LC–NMR, LC–DAD UV and LC–ESI(–)-MS/MS was used [89]. The two partly co-eluting peaks of interest, hyperoside and isoquercitrin –which are the 3-*O*-galactoside and 3-*O*-glucoside of quercetin, respectively – could not be identified with LC–MS/MS only, because they have the same molecular mass and show identical fragmentation behaviour. LC–NMR allowed unambiguous identification because of the differences in the spectra of the sugar moieties. Stopped-flow LC–NMR required scan times of several hours to record useful spectra for injected analyte masses of 10–50 μg. It is interesting to add that LC–MS/MS experiments in H_2O and D_2O were performed to determine the number of exchangeable protons in the molecules, which are not visible in NMR. This enabled the determination of the number of hydroxy groups of each constituent.

Table 13. LC–NMR studies of flavonoids.

NMR type	Vol. flow cell (µl)	LC eluents	Inj. vol (µl)	Sample	Solvent	Flavonoid constituents	Reference
Unity Innova 500 MHz*	60	D_2O, MeCN, TFA	10-20 (1 mg extr. inj. on col.)	*Gentiana ottonis*		swertisin, isoorientin	156
DRX-500**	120	D_2O, MeCN, AcOH	20	*Hypericum perforatum* (St. John's worth)	D_2O CD_3OD	quercetin–galacturonide, hyperoside, I3-II8-biapigenin	89
DRX-500**	120	D_2O, MeCN, TFA	100	apple peel		5 quercetin glycosides, 1 quercetin diglycoside	157
AMX 600**	120	D_2O, MeOH	10	leaves *Sorocea bomplandii*	MeOH	1 kaempferol–diglycoside, 1 kaempferol–triglycoside, 1 quercetin–diglycoside	198
Unity Innova 500 MHz*	60	D_2O, MeCN	not specified	roots *Erythrina vogelii*	MeOH	prenylated isoflavones, isoflavanones	199
Avance 400**	120	CD_3CN, D_2O 0.1% TFA	100	leaves *Trifolium pratense* L. (red clover)	CD_3OD D_2O	2 formononetin–glucoside–malonates (isomers), 2 biochanin A–glucoside–malonates (isomers)	44
DMX 500**	120	D_2O, MeCN, TFA	60-100	*Lycopersicon esculentum* (tomato)	CD_3OD D_2O	2 kaempferol–diglycosides, 2 dihydrokaempferol–hexosides, 2 kaempferol–glycosides, rutin, naringenin–7-*O*-glucoside, naringenin, chalcone	174
DPX 400** AV-600**	120 20	CD_3CN, D_2O 0.1% FA	20	oregano	Me_2O	taxifolin, aromadendrin, eriodictyol, naringenin, apigenin	36

* Varian (Palo Alto, CA, USA). ** Bruker (Rheinstetten, Germany). Abbreviations: FA: formic acid, AcOH: acetic acid, MeOH: methanol, MeCN: acetonitrile.

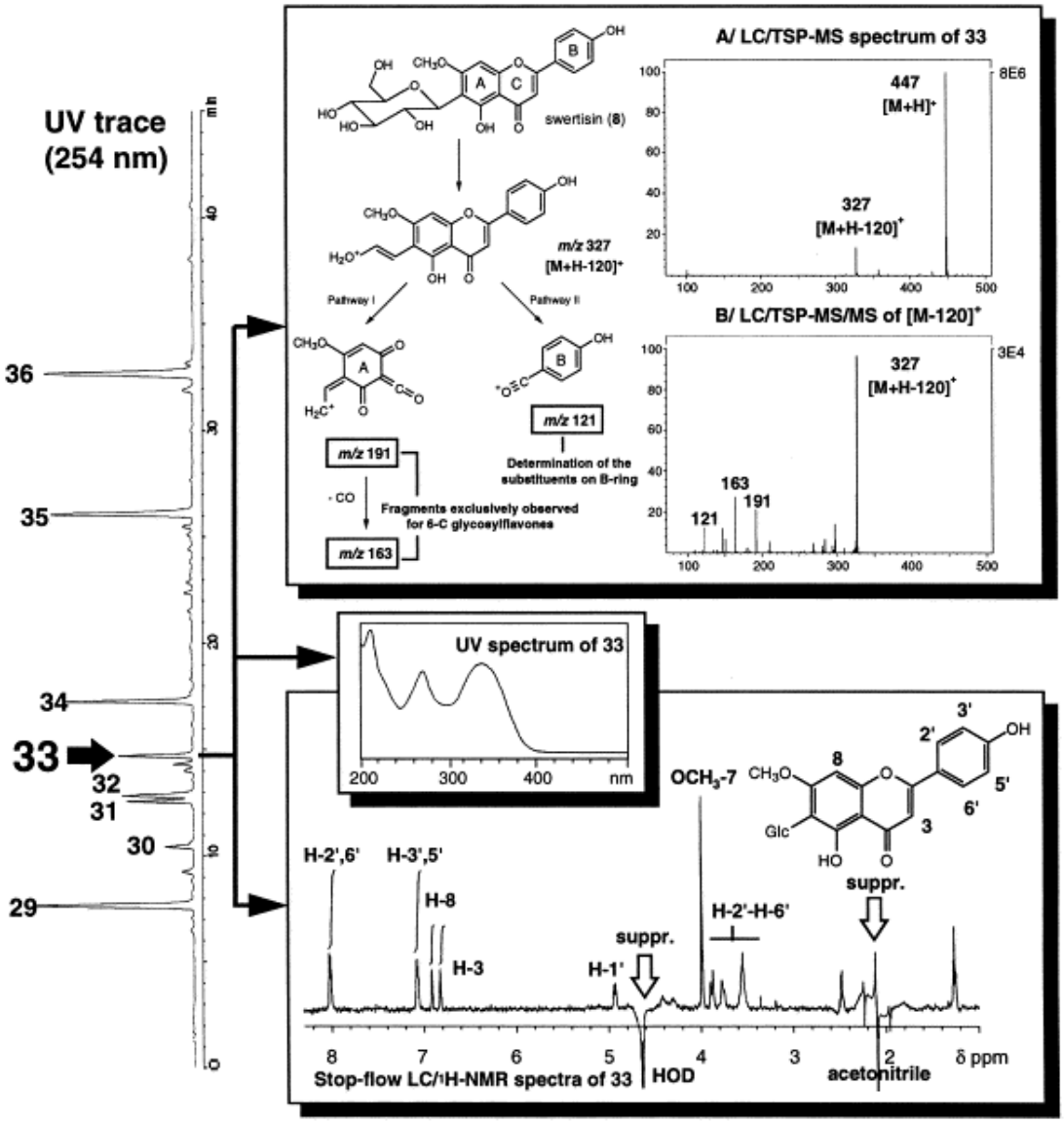


Fig. 17. LC–UV_{254} chromatogram of *Gentiana ottonis* with the ^{1}H-NMR, DAD UV, MS and MS/MS spectra of swertisin (peak 33). TSP, thermospray interface [156].

Flavonoid glycosides in apple peel were successfully identified by using the same combination of techniques as in the previous example [157]. Five quercetin glycosides and one quercetin diglycoside were recognized with LC–MS/MS, but no complete structure elucidation was provided. The glycosidic nature of the flavonoids in the extract was determined with LC–NMR, because the different sugar moieties each have their typical resonances. The sugar linkage position was derived from a comparison with reference compounds. For the least abundant compound in the extract, rutin (concn., 40 μg/ml), the time needed to record spectra with a reasonable signal-to-noise ratio for a 100 μl injection was approx. 1.5 h.

LC–DAD/UV–SPE–NMR was used in combination with on-line radical scavenging detection for the identification of radical scavenging compounds in extracts of *Rhaponticum carthamoides* [158]. A combination of on-line recorded ^{1}H-NMR spectra, MS/MS fragmentation and exact mass data were used to determine basic structures and elemental composition, while HMBC experiments were performed off-line to determine the sugar-linking type, after trapping the compounds of interest up to three times on separate SPE cartridges and combining the eluates. Without any prior off-line chromatographic steps, five flavonoid–β-glucopyranosides were identified, of which two had a 6″-*O*-acetyl group.

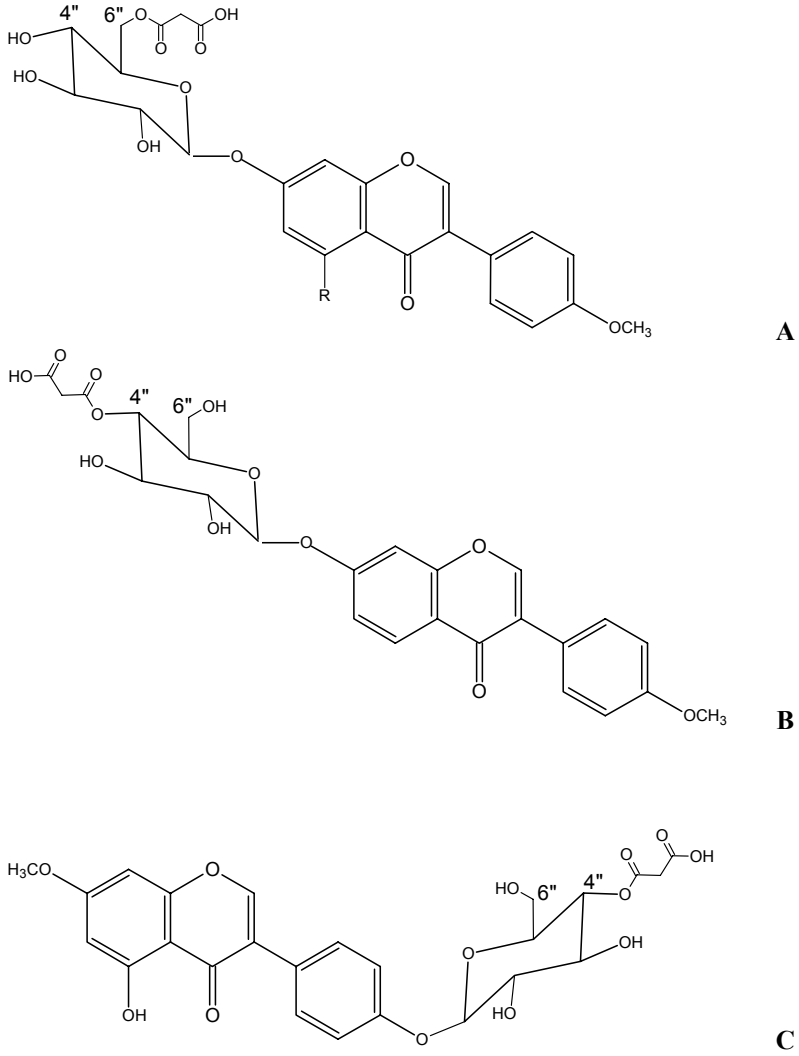


Fig. 18. Structures of: (A) formononetin–7-*O*-β-D–glucoside-6″-*O*-malonate (FGM, R=H) and biochanin A–7-*O*-β-D–glucoside-6″-*O*-malonate (BGM, R=OH); (B) formononetin–7-*O*-β-D–glucoside-4″-*O*-malonate (FGMi); and (C) 5-hydroxy-7-methoxyisoflavone–4′-*O*-β-D–glucoside-4″-*O*-malonate (BGMi).

In a recent study of de Rijke *et al.* on the flavonoid constituents of a red clover extract, stopped-flow LC–NMR and stand-alone NMR were used to identify structural

isomers that could not be distinguished on the basis of MS/MS information [44]. By combining the information provided by MS/MS, ^{1}H-NMR, COSY and NOESY spectra recorded for two sets of isoflavone–glucose–malonate isomers, not only the positions of the glucose moieties on the flavonoid aglycones, but also those of the malonate moieties on the glucose groups were determined. One set of isomers only differed in the substitution position of the malonate group on the glucoside ring, but rather unexpectedly – because the two pairs of isomers were thought to be mutually closely similar – for the other set of isomers the position of the glucose group was also different. Their structures are shown in Fig. 18. High mg/l concentrations of the analytes had to be used to record satisfactory NMR spectra on a 400 MHz instrument (1052 scans per peak).

In all of the studies quoted in Table 13 conventional-size LC was used. Currently, much effort is devoted to the development of micro- or even nano-LC–NMR. This is an attractive development since expensive deuterated solvents, which are required to suppress the eluent background signals in ^{1}H-NMR, can now be used more easily. Much attention is also paid to probe design, in order to improve sensitivity. The recently developed cryoflow NMR probe [159] that cools the receiver coils to cryogenic temperatures to improve the signal-to-noise ratio of the NMR spectra has been applied for the analysis of an oregano extract [36]. Five flavonoids were identified using an LC–UV–SPE–NMR–MS set-up. However, scan times were not noticeably shorter than in the LC–NMR studies quoted above. In other words, a clear demonstration of the potential of cryoflow LC–NMR is urgently awaited.

1.5 Conclusions

In the field of flavonoid analysis, it is good to distinguish the determination of only aglycones – frequently a rather limited number of well-known target compounds, and present in relatively high concentrations in the sample type of interest – and the detection-plus-identification of large numbers of aglycones and their conjugates, often present at the trace level, in *e.g.* plant material. In the former, target-type, analysis, sample treatment is directed at conversion of any conjugates present into the corresponding aglycones. Conditions can therefore be fairly harsh, and simple procedures can be used. LC–UV will often give fully satisfactory (quantitative) results; nevertheless, LC–single-stage MS is making headway because it provides more selective and, consequently, more reliable data. If screening of (a large number of) samples is an important aspect, planar chromatography can be considered an efficient tool.

When, on the other hand, known as well as unknown flavonoid conjugates have to be preserved intact, sample-preparation conditions have to be sufficiently mild. In addition,

optimization of the LC conditions is required to create an efficient separation of the much larger number of analytes of interest. Proper provisional identification requires the use of tandem-MS techniques as provided by triple-quadrupole and ion-trap instruments. For a more complete structure elucidation of conjugates, the complementary information derived from LC–NMR is indispensable.

Today, one may well conclude that – primarily as a result of recent, and still on-going developments in tandem-MS detection – the analytical tools for the reliable detection, identification and quantification of even low concentrations of flavonoids, *i.e.* aglycones and conjugates, are available. Further developments may be expected with regards to miniaturization, that is, the coupling of micro- and/or nano-LC, and also CE, to tandem-MS and NMR instruments: This should facilitate the analysis of minute samples, and help to create better operating conditions for NMR detection. However, it has to be added that progress in this area has been slower than was expected several years ago.

There is, to our opinion, little doubt that, in the near future, LC with tandem MS detection will continue to play a dominant role in flavonoid analysis. Next to excellent sensitivity, this technique can provide structural information based on various C-ring cleavages (including RDA fragmentation), and loss of small fragments. It will be an aspect of interest to determine more clearly than has been done until now, to which extent such fragmentation pathways can be used as 'indicators' for the presence of specific flavonoids. Generally speaking, attention will, and should, be shifted from analytical method development to more in-depth, and more application-oriented studies, for example in natural product research and in work on the unravelling of metabolic pathways in the human body – with the highly interesting anti-oxidant properties of flavonoids as a key issue.

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1.7 Scope of the thesis

The present research is part of the project 'Wetlands in the Randstad', a cooperation between the Faculty of Earth and Life Sciences, the Faculty of Exact Sciences, the Faculty of Economics and the Institute of Environmental Studies of the Free University, which focuses on the effects of flooding or '*vernatting*' of polder and wetland areas in the densely populated Randstad area of the Netherlands, to restore their ecological value. The aim of our research was to develop adequate methods of analysis for flavonoids including their conjugates in complex matrices, in particular wetland plants. In the end, these methods should be used to study the potential of flavonoids as indicators for ecological changes due to *vernatting*. The flavonoids were selected because, contrary to *e.g.* lignins and tannins, they are chemically well-defined compounds. Furthermore, they possess chromophoric groups so that they can be studied by UV/Vis spectrometry and exhibit useful fragmentation in mass spectrometry; some flavonoids show native fluorescence.

In this thesis, flavonoids in plants of the Leguminosae family, which commonly occur in Dutch wetland areas, are studied using various hyphenated analytical methods. The study comprises (i) optimization of extraction procedures, with emphasis on the stability of selected flavonoids; (ii) research on UV absorbance, fluorescence and NMR spectroscopy as well as (tandem) mass spectrometry of flavonoids in order to find optimal detection conditions and, as a first application, (iii) a study of the effect of *vernatting* which focuses on the behaviour of the main flavonoids in the leaves of red clover (*T. pratense*) under waterlogging conditions.

After the introduction presented in **Chapter 1**, in which an extensive overview is given of the state-of-the-art of various analytical methods available to determine flavonoids in a variety of matrices – and in which the aim of the study is outlined – the first item of interest in the analysis of flavonoids in plants, the development of an adequate extraction procedure, is presented in **Chapter 2**. This discusses the determination of isoflavones, their glucosides and glucoside–malonates in red clover using reversed-phase liquid chromatography (LC) coupled to atmospheric pressure chemical ionisation mass spectrometric (APCI-MS), UV and fluorescence detectors. As regards hydrolysis and storage conditions of methanolic extracts, storage is best done at –20°C to prevent degradation of flavonoid–glucosides and glucoside–malonates for at least 1–2 weeks, while hydrolysis should preferably be performed over a 2-h period at 83°C, without acidification. Under such conditions the glucoside–malonates, formononetin–7-*O*-β-D-glucoside–6″-*O*-malonate and biochanin A–7-*O*-β-D-glucoside–6″-*O*-malonate, are quantitatively converted into the corresponding glucosides while decomposition of the latter is still negligible. Hydrolysis at 83°C in the presence of acid provides a way to obtain additional information on the flavonoid distribution. In LC-separated

fractions the malonates are most stable when stored at low temperature after evaporation to dryness. The concentrations of eight major isoflavones were found to range from 0.04 to 5 mg/g leaves.

Chapter 3 discusses the fluorescence behaviour of nineteen flavonoids. The number of compounds exhibiting native fluorescence is very limited. Three isoflavones, *i.e.*, formononetin, ononin and daidzein showed fluorescence. Interestingly, they were found to exhibit unexpectedly large Stokes' shifts (difference between excitation and emission maxima), most likely due to a change of the structure of the molecule from non-planar in the S_0, to planar in the S_1, state. Lifetime measurements were carried out using time-correlated single-photon counting spectroscopy to further characterize the mechanism. Large Stokes' shifts provide a high selectivity, so that the fluorescent isoflavones can easily be detected in plants by means of LC with a methanol–water gradient and fluorescence detection with a large wavelength difference between λ_{ex} and λ_{em}.

In **Chapter 4**, the analytical performance of four modes of LC–MS (APCI, ESI; positive ionization and negative ionization) is compared for two mass spectrometers, a triple-quadrupole and an ion-trap instrument, with fifteen flavonoids as test compounds. Two organic modifiers, methanol and acetonitrile, and two buffers, ammonium acetate and ammonium formate, were used. In general, the eluent combination methanol–ammonium formate buffer of pH 4.0 gives the highest responses for the flavonoids, with APCI(–)-MS in first, and ESI(–)-MS in second place. The responses of the four ionization modes and the fragmentation reactions in MS/MS are essentially the same on the ion-trap and triple-quadrupole instruments. Under optimum conditions full-scan LODs of 0.1–30 mg/l were achieved in the APCI(–) mode, and up to two-order response differences were found both between analytes and between modes of ionization. LC–MS yields characteristic fragmentation patterns of the flavonoids and provides information of the nature of the aglycon, the presence of sugar moieties, malonate groups and methyl substituents. Generally, three main ions are formed, $[M–H]^-$ or $[M+H]^+$, $[M–15]^-$ and $[M–2Gly]^-$ or $[M–Gly]^-$. Furthermore, retro Diels-Alder fragments due to cleavage of the C-ring are formed. Formic acid adduct formation generates $[M+45]^-$ ions and occurs in the negative ionization modes only. MS/MS does not provide much additional information in most instances: generally speaking, the same fragments are observed as in full-scan MS. The main fragmentations observed in the MS^n spectra of the ion-trap, and the tandem MS spectra of the triple-quadrupole instrument generally were the same. The advantage of the former approach is the added possibility to ascertain precursor → product ion relationships.

Chapter 5 studies the relative flavonoid content of several plants of the Leguminosae family. LC on a C_{18}-bonded silica column with a methanol–ammonium formate gradient was used to determine the main flavonoids in leaves of four different plant species. The detection

modes were DAD UV, fluorescence and (tandem) MS. LC DAD–UV was used for general screening, sub-classification, and calculation of total flavonoid concentrations. LC–FLU was used to identify isoflavones on the basis of their native fluorescence. Most structural information regarding aglycons, sugar moieties and acidic groups was derived from LC–APCI(–)-MS in the full-scan and extracted-ion modes. Again, MS/MS did not provide much additional information because the same fragments were observed as in full-scan MS. In *T. pratense* L. (red clover) and *T. repens* L. (white clover), the main constituents were flavonoid–glucoside–(di)malonates, while *T. dubium* L. (small hop clover) and *L. corniculatus* L. (common bird's-foot trefoil) mainly contained flavonoid–(di)glycosides. Satellite sets comprising an aglycon, the glucoside and glucoside–malonates or –acetates, were abundantly present in *T. pratense*, where some thirteen such sets were identified. Quite differently, at best one satellite set was found in the leaves of the other three plants. Generally speaking, the main aglycons and sugars in the four plant species are surprisingly different – for example, formononetin and biochanin A in the case of *T. pratense*, but kaempferol in the case of L. *corniculatus*. Similarly, as regards the sugars, glucose is most prominent in *T. pratense*, but glucose and rhamnose in the case of *T. dubium*. In addition, while the results for *T. pratense* are similar to those reported in the literature, there is little agreement in the case of the other species. Finally, total flavonoid contents ranged from 50–65 mg/g leaves for *L. corniculatus* and *T. dubium*, to 15 mg/g for *T. pratense* and only 1 mg/g for *T. repens*. At the end of this study, one problem – the elucidation of the detailed structures of some of the isoflavone–glucoside–malonate isomers found in *T. pratense* – still had to be answered. This was done in Chapter 6.

Chapter 6 illustrates the potential of LC–NMR as a complementary technique to LC–MS(/MS). Together with off-line two-dimensional NMR, LC–NMR was used to obtain structural information to distinguish the main isoflavone–glucoside–malonate isomers in extracts of leaves of *T. pratense* L., since the LC–MS(/MS) studies of Chapter 5 did not provide sufficient information. Matrix solid-phase dispersion (MSPD) was applied as sample preparation technique to obtain the sufficiently high analyte concentrations required to perform LC–NMR. Stop-flow reversed-phase LC–NMR was conducted using a gradient of deuterated water and deuterated acetonitrile. Off-line COrrelation SpectroscopY (COSY) and Nuclear Overhauser Enhancement SpectroscopY (NOESY) experiments were carried out to determine the positions of the glucose moiety on the flavonoid aglycone, and of the malonate moiety on the glucose. Based on the fragmentation patterns observed in MS/MS and the NMR spectra, the two formononetin–glucoside–malonate isomers were identified as 7-*O*-β-D-glucoside–6″-*O*-malonate and 7-*O*-β-D-glucoside–4″-*O*-malonate; that is, they differ only in the substitution position of the malonate group on the glucoside ring. The biochanin A

glucoside–malonate isomers, however, have quite different structures. The main and later eluting isomer is biochanin A–7-*O*-β-D-glucoside–6"-*O*-malonate, and the minor isomer, 5-hydroxy-7-methoxyisoflavone–4′-*O*-β-D-glucoside–4″-*O*-malonate: the positions of the methoxy group and the glucoside–6″-*O*-malonate group on the flavonoid skeleton are interchanged.

It was concluded in Chapter 5 that among the four plant species considered *T. pratense* contained the highest flavonoid concentrations. This plant was therefore selected to be further studied under waterlogging conditions. In **Chapter 7** the optimized LC–UV–APCI(–)-MS method was used to study the effect of disturbed root nodulation on qualitative and quantitative changes in the composition of the main isoflavonoid–glucoside–malonates, glucosides and aglycons in the plant leaves. Isoflavonoids are involved in the regulation of root nodule activity and the establishment of mycorrhizal association. It was found that, in response to waterlogging, the concentrations of the free aglycon, biochanin A, and the conjugates biochanin A–7-*O*-glucoside–malonate, biochanin A–7-*O*-glucoside, and genistein–7-*O*-glucoside in the leaves increased 2–3-fold after a lag period of three weeks, probably as a result of disturbed root nodulation. The other isoflavones that were detected – the free aglycon, formononetin, and the conjugates formononetin–7-*O*-glucoside–malonate and formononetin–7-*O*-glucoside – did not show any significant changes related to waterlogging. After restoring the normal soil water conditions, the concentrations of biochanin A and its two conjugates rapidly returned to the initial values, while the concentration of genistein–7-*O*-glucoside remained high. This suggests that the last-named flavonoid may be a useful chemical indicator to study waterlogging influences. However, further ecological research is needed to confirm this suggestion.

2 Determination of isoflavone glucoside malonates in *Trifolium pratense* L. (red clover) extracts: quantification and stability studies[1]

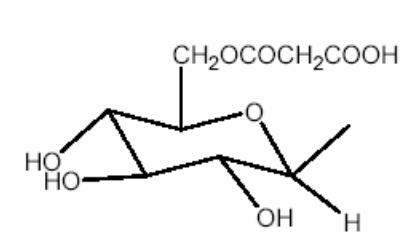
Summary

Isoflavones, their glucosides and their glucoside malonates were determined in red clover leaf extracts using reversed-phase LC coupled to atmospheric pressure chemical ionisation mass spectrometry, UV and fluorescence detectors and the stability of the malonates was investigated. Extracts can be stored at least 1–2 weeks at –20°C without loss of malonates. In LC-separated fractions the malonates are most stable when stored at low temperature after evaporation to dryness. The concentrations of eight major isoflavones ranged from 0.04 to 5 mg/g leaves.

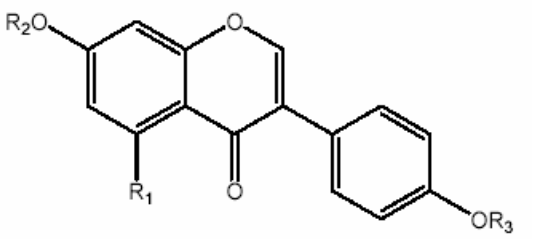
[1]E. de Rijke, A. Zafra-Gómez, F. Ariese, U. A.Th. Brinkman, C. Gooijer, *J. Chromatogr. A* 931 (2001) 55.

2.1 Introduction

Flavonoids are one of the most characteristic classes of compounds in higher plants. Many flavonoids are present as flower pigments in most angiosperm families. However, their occurrence is not restricted to the flowers but includes all parts of the plant. From a chemical point of view they comprise a wide group of structurally related compounds with a chromane skeleton provided with a phenyl substituent at the C_2 (flavones) or C_3 (isoflavones) position; the latter subclass is depicted in Fig. 1. Flavonoids are usually found in plants as glycosides, *i.e.* provided with sugar substituents such as galactose, rhamnose or glucose, or glycoside malonates. The malonates are of biological interest because the plant can use this conjugated form to store the less soluble flavonoid aglycons. Upon microbial infection, the aglycons are generated from these precursors by hydrolysis of the stored form [1]. Isoflavonoid phytoalexins play an important role in fungal infection, but isoflavonoids are also present in legumes, where they accumulate in the vacuoles either in their glycoside or their 6”-*O*-malonylated form [2]. Flavonoids may offer several benefits to human health, including antioxidant activity, metal chelation [3,4] and anticarcinogenic, antiallergic and antiviral effects [5]. They have also been shown to stimulate the immune system and to prevent nitration of tyrosine [6].



6”-*O*-malonylglucosyl



isoflavone structure

Name		R_1	R_2	R_3	MW
Daidzein	D	H	H	H	254
Daidzin (1)	DG	H	7-*O*-β-D-glc	H	416
Genistein	G	OH	H	H	270
Genistin (2)	GG	OH	7-*O*-β-D-glc	H	432
Formononetin (7)	F	H	H	CH_3	268
Ononin (3)	FG	H	7-*O*-β-D-glc	CH_3	430
Formononetin–7-*O*-β-D-glucoside–6”-*O*-malonate (4)	FGM	H	7-*O*-β-D-glc-6”-*O*-mal	CH_3	516
Biochanin A (8)	B	OH	H	CH_3	284
Sissotrin (5)	BG	OH	glc	CH_3	446
Biochanin A–7-*O*-β-D-glucoside–6”-*O*-malonate (6)	BGM	OH	7-*O*-β-D-glc-6”-*O*-mal	CH_3	532

Fig. 1. The structures of the ten isoflavones studied and their abbreviations used in this paper; glc, glucoside; mal, malonate. The numbers in parentheses refer to the peaks in the chromatograms of Fig. 2.

Red clover (*Trifolium pratense* L.), also known as meadow, creeping or cow clover, is an important forage plant which grows in many parts of the world. It is an important feeding material for sheep and cattle and a health products for humans as medicinal herb for the treatment of eczema and psoriasis [7,8]. Many flavonoids, as well as glycoside and glycoside malonate derivatives of flavonoids have been identified in red clover and other species using reversed-phase LC (RPLC) coupled with MS detection [7,9,10]. Most workers have identified the same major flavonoids. However, in most studies no attention is paid to quantification and consequently, sample pretreatment, which is usually done by means of hydrolysis, is mentioned only briefly. Since it is our goal to study flavonoid profiles in wetland plants to use these as indicators for stress in wetland ecosystems, quantitative aspects of flavonoid analysis are very important. Therefore, in this work the stability and hydrolysis of red clover leaf extracts is investigated. For two selected major constituents of the extracts, the isoflavone glucoside malonates FGM and BGM (*cf.* Fig. 1), stability and degradation pathways were investigated using RPLC–UV, with the optimization of sample handling conditions as main objective. Subsequently, under optimised conditions, ten major isoflavones were determined (and their presence confirmed) by using RPLC–UV with atmospheric pressure chemical ionisation (APCI)-MS and fluorescence detection (FLU) for identification and additional confirmation.

2.2 Experimental

Materials

Daidzin, daidzein, genistin, genistein, formononetin and ononin were purchased from Roth (Karlsruhe, Germany), biochanin A and sissotrin from Indofine Chemical (Somerville, NJ, USA). Methanol, formic acid and HCl were from J.T. Baker (Deventer, the Netherlands), 4-hydroxy-1-naphthalenesulphonic acid and ammonium formate from Aldrich (Steinheim, Germany), tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, the Netherlands). Extracts were made using fresh leaves of T. pratense from plants collected in a field (Ouddorp, the Netherlands) and grown in a greenhouse.

Extraction and isolation procedures

The procedure for the extraction of flavonoids from red clover was adapted from Toebes *et al.* [11]. 500 mg of fresh leaves were extracted in an ultrasonication bath for 60 min at room

temperature with 10 ml of methanol-water (9:1, *v/v*) and 3 ml of aqueous 350 mM Tris buffer. The extracts were filtered over a 0.45 µm filter before pretreatment and/or injection.
Fractions containing the two most dominant peaks, FGM and BGM, were collected after LC separation by collecting 1 ml of column eluent, at their peak maximum on the UV detector. The samples were hydrolysed by heating the filtered extracts in a sealed vial up to 4 h at 83 ºC in the presence or absence of concentrated HCl.

RPLC–UV and -FLU

LC was performed on a two-pump system equipped with a dynamic mixer and a manometric module (Gilson Medical Electronics, Villiers-le-Bel, France). UV and fluorescence data were obtained with a Hewlett-Packard Series 1050 UV detector (set at 265 nm) and an Applied Biosystems (Foster City, CA, USA) Model 980 programmable fluorescence detector (λ_{ex} = 250 nm, emission filter at 418 nm). A 250 x 4.6 mm I.D. 5 µm Zorbax SB-C_{18} column was used. The eluent consisted of a mixture of methanol and aqueous 10 mM ammonium formate buffer, pH 4.0. All solvents were filtered and degassed with helium before use. The LC gradient is shown in Table 1. The flow was 1.0 ml/min and the injection volume, 10 µl.

Table 1. LC gradient used for isoflavone analysis.

Time (min)	% methanol	% buffer
0	30	70
5-10	45	55
15	50	50
25	55	45
30	60	40
35-40	99	1
42	30	70

RPLC–MS

Analyses were performed on a Shimadzu (Princeton, NJ, USA) LC system, consisting of two LC-10A LC pumps, a DGU-14A degasser, a SIL-10AD auto-injector, a SCL-10A system controller unit and a SPD-10A UV detector (set at 265 and 290 nm), coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The same gradient as in LC–UV was applied. APCI-MS spectra were acquired in the negative ion mode in the *m/z* range of 150-650. Only in an exceptional case, the positive ion mode (also in the *m/z* range 150-650) was shown to be more favourable (see Fig. 6 below). The source and probe temperatures were maintained at 150 ºC and 450 ºC, respectively, and the activation energy at 30%. The LC flow was directed into the mass spectrometer without stream splitting.

2.3 Results and Discussion

The two main isoflavones in red clover are FGM and BGM, which have been characterized in several MS-based studies as isoflavone glucoside–malonates [2,7]. They are subject to decomposition according to:

Formononetin-7-*O*-β-D-glucoside–6”-*O*-malonate (FGM) → ononin (FG) → formononetin (F) (1)

and

Biochanin A-7-*O*-β-D-glucoside–6”-*O*-malonate (BGM) → sissotrin (BG) → biochanin A (B) (2)

Below, first of all LC conditions are presented under which the above decomposition is expected to be largely absent. Next, the hydrolysis of FGM and BGM is studied under either mild or harsh conditions and their stability is investigated. Finally, quantification of the analytes of Eqs. 1 and 2 and, also, the isoflavone glucosides, daidzin and genistin, and their respective aglycons, daidzein and genistein, was performed by RPLC–UV with identification and additional confirmation by RPLC–APCI-MS and fluorescence.

LC separation

Since the present study focuses on identification as well as quantification, we used gradient LC conditions, which are compatible with UV as well as MS detection. The somewhat complicated gradient of Table 1 was found to be fully satisfactory in this respect. Its optimisation will be discussed in detail in a parallel paper [12]. Here it should be noted that contrary to Lin *et al.* [7] who used an acidic eluent (water and acetonitrile containing 0.25% acetic acid), we preferred aqueous ammonium formate at pH=4.0. In our experience such conditions are quite suitable for MS detection by means of, *e.g.*, APCI or electrospray ionsiation (ESI) in the negative mode.

The LC–UV, LC–MS and LC–FLU chromatograms of a red clover extract — independently recorded, using different experimental set-ups — are shown in Fig. 2. The LC–UV chromatogram was recorded at 265 nm, a good compromise wavelength for the isoflavones of Fig. 1, which all have absorption maxima in the 255–270 nm range. For FGM and BGM no standards were available but, most probably, their absorption characteristics hardly differ from those of FG and BG, respectively, since the chromophoric skeletons will hardly be influenced by the loss of the malonate group.

The LC–MS chromatogram serves to check whether in Fig. 2A indeed an isoflavone profile is recorded with other sample constituents playing a minor role. Comparison of the

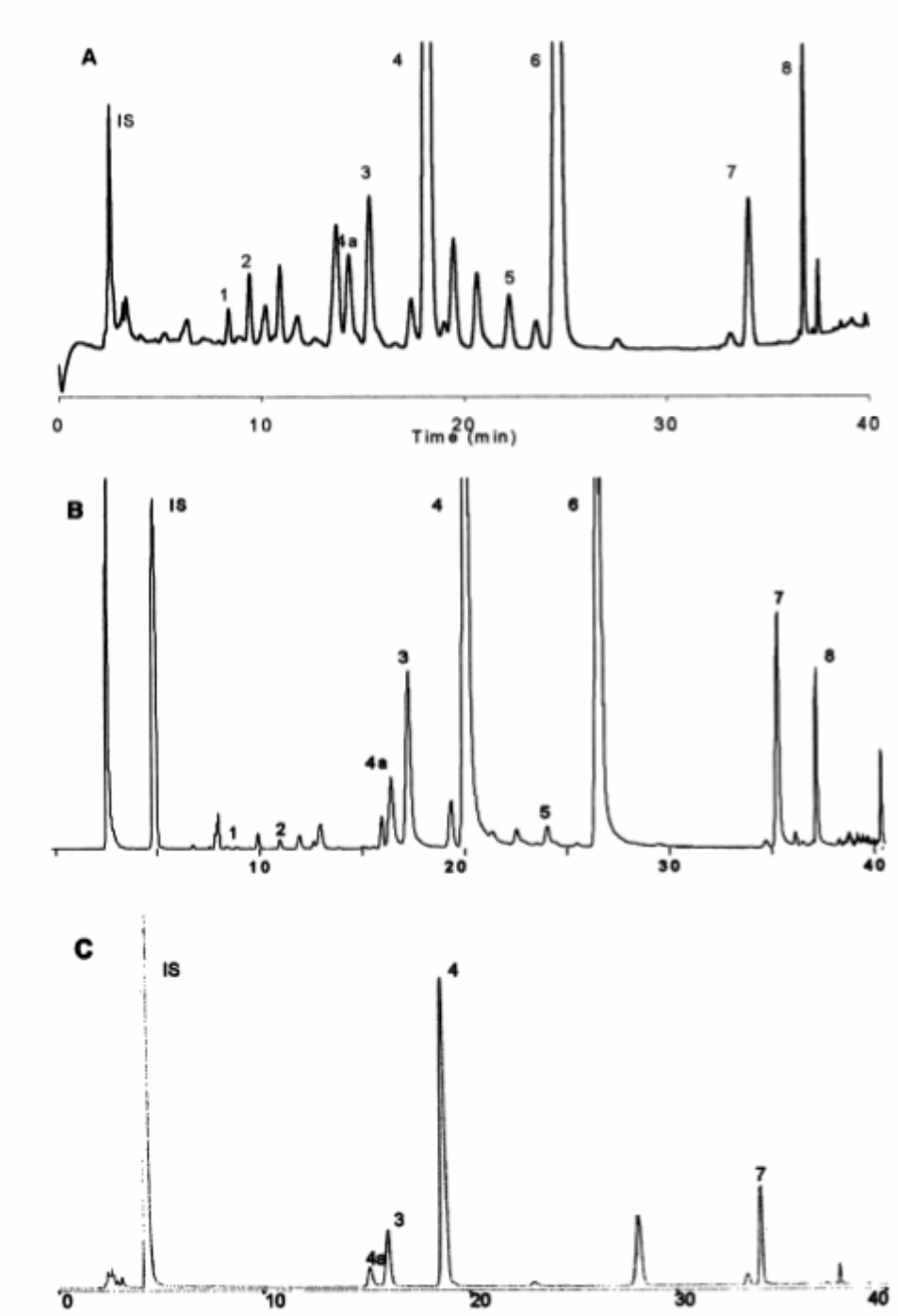


Fig 2. Independently recorded (A) LC–UV_{265}, (B) LC–APCI MS (negative mode; full scan) and (C) LC–FLD chromatograms of T. Pratense L. extract. Peak 4a is an FGM isomer, see text. Peak numbers refer to Fig. 1. The small peaks 1 and 2 in B were recognized in SIM. Vertical axes are in relative units.

two chromatograms shows that the profiles are similar, as is illustrated by the peak numbers referring to the isoflavones listed in Fig. 1. With LC–MS, 24 peaks were recorded (not all visible in Fig. 2B) using selected ion monitoring (SIM). More importantly, they could all be attributed to flavones, as will be discussed in more detail in a forthcoming paper [12];

these results generally agree with those of Lin *et al.* [7]. The intense peaks in the LC–UV chromatogram eluting at 18.2 and 24.6 min, which also dominate the LC–MS chromatogram, can be attributed to FGM and BGM, respectively, the compounds of main interest in the present study. The LC–FLU chromatogram (Fig. 2C) provides additional selectivity. As is to be expected, most isoflavones do not show native fluorescence. Only five prominent peaks can be seen in the fluorescence chromatogram. Three of them can be attributed to FG (3), FGM (4) and F (7). Peak 4a is — also in view of its mass spectrum — attributed to an isomer of FGM. The peak at 28 min probably is pratensein, but this could not be confirmed because no pratensein standard was available.

Hydrolysis of FGM and BGM

In the literature quantification of flavonoid glycosides and glycoside malonates has not received much attention, although there are some exceptions [2,10,13]. In these studies quantification is simply based on the formation of aglycons after hydrolysis, and speciation is not considered. In this paper we use FGM and BGM to study the hydrolysis process (*cf.* Eqs. 1 and 2) in some detail. In order to do rapid screening of many samples, the hydrolysis procedure of choice should be efficient and not too time-consuming. LC fractions containing only FGM and BGM were collected to prevent interfering reactions from other sample constituents as much as possible. These fractions were hydrolysed by heating without and with acidification (*cf.* ref [7]).

Heating without acidification. FGM and BGM fractions prepared as described in Section 2.2 were heated at 83^{o}C. Injections of the (partly) hydrolysed fractions were made every 30 min over a period of 2 h. Changes in the concentrations of BGM, FGM, ononin (FG), sissotrin (BG), formononetin (F) and biochanin A (B) were monitored on the same LC system as used above. Prior to injection 20 µl of a 1000 µg/ml 4-hydroxy-1-naphthalene sulphonic acid solution was added to 200 µl of each fraction as an internal standard (IS). Typical results are shown in Fig. 3. Responses are expressed as the ratio of the peak areas of the analytes and the IS. The data show that both FGM and BGM completely disappear within 2 h. The corresponding glucosides, FG and BG, show a simultaneous concentration increase. Since the UV signals i.e., the concentrations of the aglycons F and B remain essentially constant at a very low level — there are always some aglycons present in the sample that are formed during extraction — one can conclude that, in the absence of acid, quantitative conversion of the parent compounds into the relatively stable glucosides FG and BG can easily be achieved. Heating for more than 2 h is not advisable because the data of Figs. 3A and C suggest that the glucosides then begin to decompose (which may well considerably influence results obtained after 16 h heating such as reported in ref. [7]).

Heating with acidification. Similar experiments were performed in which heating at 83°C was carried out after the addition of 40 µl of concentrated HCl to 200 µl of sample. LC analysis was done in the same way as above. Quite different results were obtained. Fig. 3 shows that, in the presence of HCl, degradation of BGM and FGM is more rapid, and is taken one step further. The parent compounds are lost within 60-90 min, but the glucosides which are formed, are subsequently rapidly converted to the aglycons F and B. This conversion is complete in about 2 h, but the aglycons themselves also show degradation, as is shown by the analyses of the 4-h-treated samples. Obviously, for quantification purposes hydrolysis in the absence of acid is to be preferred.

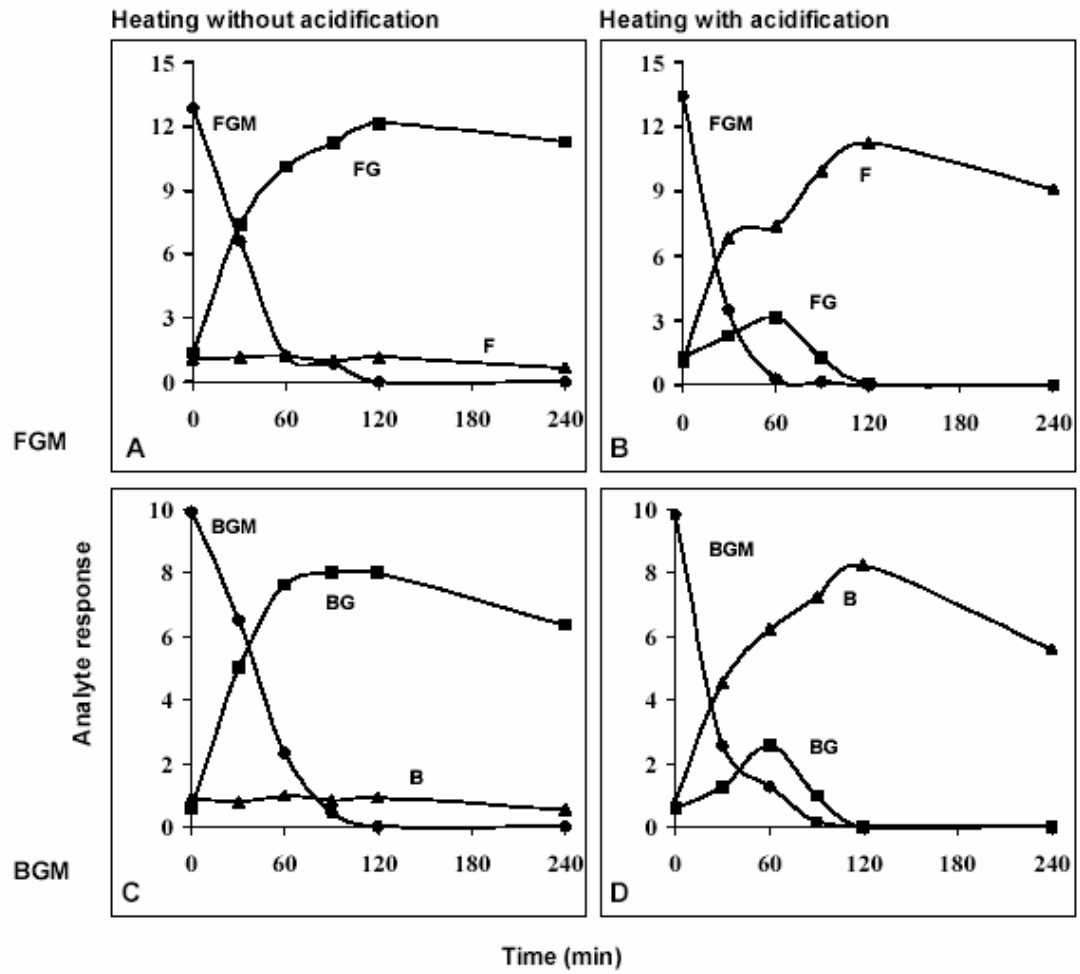


Fig. 3. Effect of hydrolysis on the degradation of FGM (A and B) and BGM (C and D): (A and C) by heating at 83°C and (B and D) by heating at 83°C in the presence of HCl. For LC conditions, see text.

Stability of FGM and BGM in extracts of leaves

For longer-term studies, it is important to know whether or not the intact malonates, FGM and BGM, are stable for prolonged periods of time, either in the extracts themselves or in the fractions collected after LC separation.

Stability in extracts. A red clover extract in methanol containing 100 µg/ml of IS was divided into three portions. One of these was stored in the freezer at –20°C and another one at room temperature. The third portion was stored in the freezer after evaporation to dryness under nitrogen. Changes in the composition of the three samples were studied over a period of two weeks and monitored by means of LC–UV. The results displayed in Fig. 4 show that, in the solution stored in the freezer, the malonates are stable for at least 1–2 weeks. However, if the sample is kept at room temperature and, somewhat surprisingly, also if it is stored dry at –20°C, degradation starts to occur almost immediately. Under these conditions, the malonate group is lost, but the glucosides remain intact. The decrease in the malonate concentrations in dried samples may be due to incomplete evaporation of the eluent, *i.e.* the continued presence of part of the buffer constituents.

In all further studies, we used solutions of extracts that had been stored at –20°C for a maximum period of 1 week.

Stability in LC-separated fractions. The LC fractions containing FGM and BGM were collected as described above. Aliquots of these fractions dissolved in the methanol-formate buffer used as LC eluent were stored in the freezer and injected at t = 0, 4, 7 and 14 days. Other portions of the same fractions were evaporated to dryness under nitrogen at room temperature. One part was redissolved in methanol immediately after evaporation and was then stored in the freezer. Another portion was stored dry in the freezer and redissolved in methanol just prior to injection. It will be clear that the latter portion had to be taken from the freezer prior to analysis and thawed; next, after dissolving it in methanol and taking an appropriate volume for injection and LC analysis, the remaining solution had to be evaporated again. That is, there was more manipulation than with the other samples. The results of Fig. 5 show that the malonates are not stable when stored at –20°C in methanol-formate buffer: within 24 h they are completely decomposed. Stability is also poor for samples dissolved in methanol after evaporation of the eluent, but complete decomposition now takes several days. The best option is to store the fractionated malonates dry and redissolve them just prior to injection. However, even then the stability is not as good as observed for the original extracts stored at low temperature (*cf.* Fig. 4). Similar results were obtained when evaporation was carried out with helium instead of nitrogen.

In summary, extracts can be stored at least 1–2 weeks at –20°C without loss of malonates. In separated LC fractions the stability is invariably poorer, with the best results being obtained for samples stored dry at –20°C. Possibly, the malonate decomposition is caused by a less than complete removal of the eluent plus buffer during evaporation. This would explain why the results are somewhat better with the repeated dissolution and evaporation procedure.

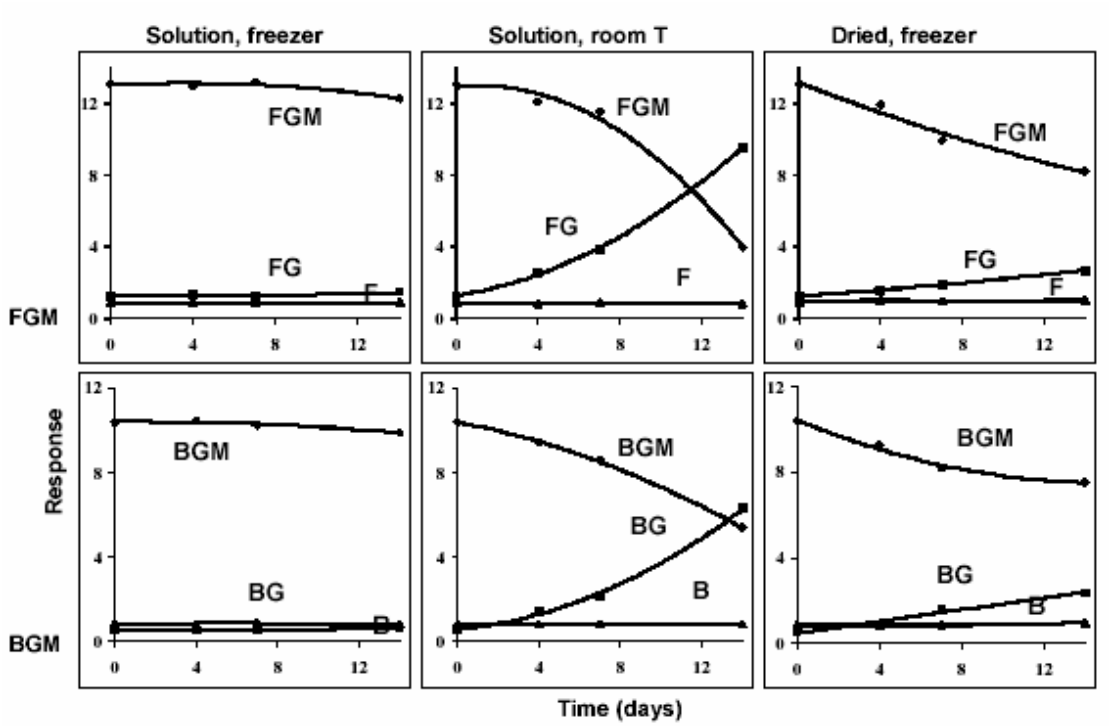


Fig. 4. Stability of FGM and BGM in a red clover extract under different storage conditions. For details and LC conditions see text.

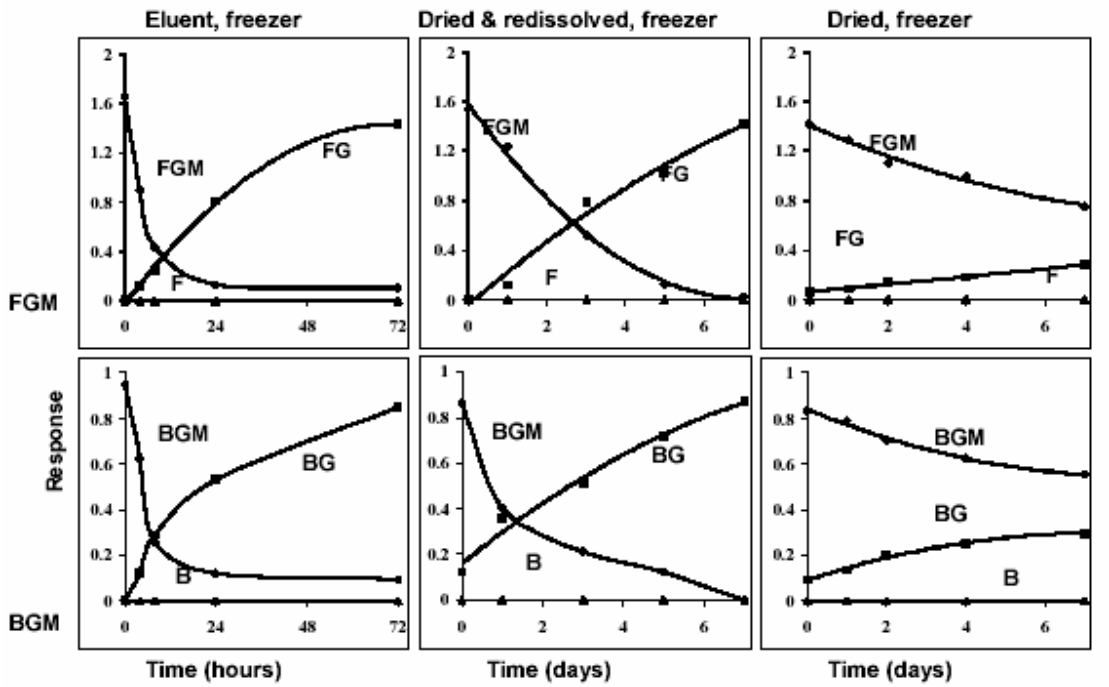


Fig. 5. Stability of FGM and BGM in LC-separated fractions under different storage conditions. For details and LC conditions see text.

Fluorescence

As noted above, ten isoflavones were selected to characterize the clover leaf extracts (cf. Fig. 1). Unfortunately, they all have the same 'chromophoric skeleton' and therefore quite similar UV absorption characteristics. In other words, UV detection provides hardly any selectivity. The situation is, however, completely different when fluorescence detection is used. In fact, the number of isoflavones that show native fluorescence is quite limited. Therefore LC–FLU is potentially useful for qualitative studies.

It was found that, from among the ten isoflavones mentioned above, FGM, FG and F emit strong fluorescence, in contrast to BGM, BG and B which are non-fluorescent (cf. Fig. 2). Daidzin (DG) and daidzein (D) deserve special attention. Standard solutions of both DG and D displayed fluorescence. However, when the DG standard was analysed by LC–MS, a much smaller peak was detected with the same mass as DG, which co-elutes on the tail of the daidzin peak but has a different fragmentation pattern (Fig. 6). Further experiments showed that it is this isomer, which is present in the DG standard at very low concentration, that causes the relatively high fluorescence signal. In fact DG itself did not show any fluorescence at all. The excitation maximum of the isomer is at 240 nm, the same as for D. As far as we know this point did not yet receive any attention in the literature.

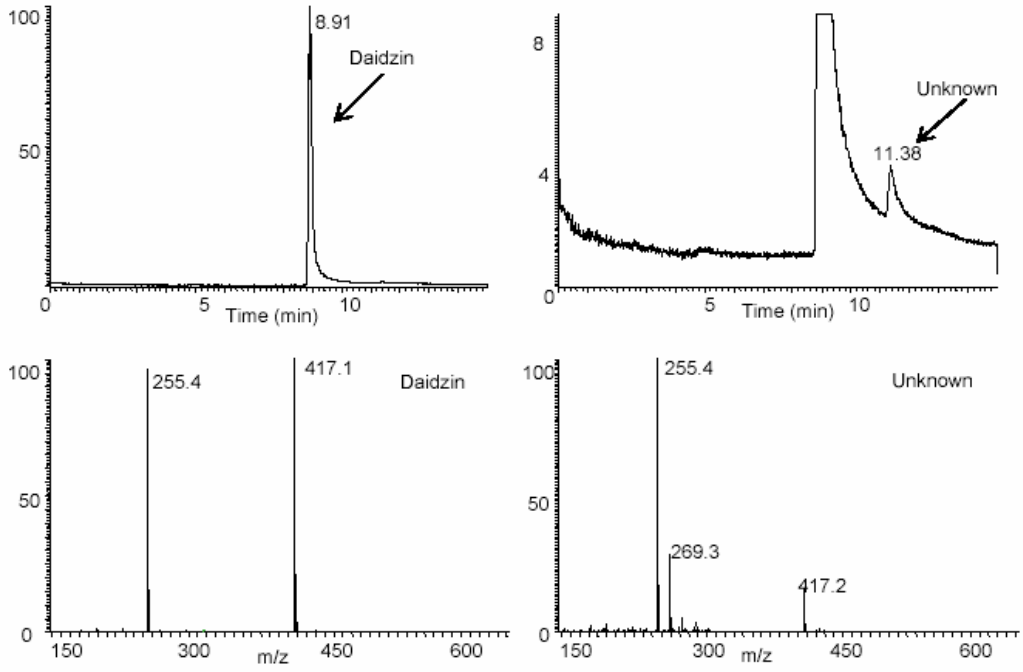


Fig. 6: LC–APCI-MS chromatogram (positive mode; full-scan) of daidzin standard (t_R 8.91) and co-eluting isomer (t_R 11.38) with corresponding mass spectra. For details see text.

Quantification

Quantification of the ten isoflavones was carried out using LC–UV. FGM and BGM, of which no standards are available, were quantified after hydrolysis to ononin and sissotrin,

respectively. Calibration plots were constructed by injecting standard solutions containing between 0.10 and 125 μg/ml of the analytes (10 data points in triplicate). R values were between 0.9998 and 0.9980. Detection limits (S/N=3; n=7) are presented in Table 2; they were between 20 and 90 μg/ml. Repeatability data are also shown in Table 2: for standard solutions all RSD values of the peak areas (n=7) were below 10%.

For the clover extracts all analyses were carried out in triplicate. Table 2 summarises the results. For the sake of convenience, the concentrations are expressed in μM, since analyte conversions (after hydrolysis) are considered. As is to be expected for a glucoside, the concentration of daidzin remains essentially the same after the non-acid hydrolysis, and becomes zero after acid hydrolysis. Daidzein was not detected in the clover extract, but was formed as a result of 1 M acid hydrolysis due to loss of the glucose group of daidzin. The same behaviour is found for the glucoside/aglycon pair genistin/genistein. The conversion yield was about 80% for the genistin/genistein pair and about 30% for daidzin/daidzein. Probably, genistein and, much more so, daidzein undergo further decomposition. Further study of this aspect was beyond the scope of the present project.

FGM and BGM were determined by subtracting the concentrations of FG and BG before non-acid hydrolysis from those found after this treatment. If heating is performed under acidic conditions, the glucosides are degraded further into their corresponding aglycons, a somewhat different situation as was explained above in the context of Figs. 3B and 3D. With the FGM/FG/F series, the final formononetin concentration of 260 μM closely matches the summed concentration of the three compounds in the initial extract (270 μM). With the BGM/BG/B series there is some discrepancy, (130 *vs*. 163 μM) which probably reflects some further degradation, also mentioned above.

Table 2. Quantification of isoflavones in clover leaves; the LOD and RSD columns refer to standard solutions.

Analytes	LOD (μg/ml)	RSD (%) of peakarea, n=7	Conc initial extract (μM)	Conc extract after heating (μM)	Conc extract after heating + HCl (μM)	Conc in leaves (μg/g)
Daidzin	35	5	1.8	1.9	0	42
Daidzein	70	5	0	0	0.6	0
Genistin	85	6	25	26	0	560
Genistein	90	7	0	0	21	0
FGM	-	-	186*	0	0	4900
Ononin	80	4	44	230	0	970
Formononetin	65	2	43	45	260	600
BGM	-	-	117*	0	0	3300
Biochanin A	50	3	23	140	0	540
Sissotrin	20	4	23	27	130	330

* Calculated values, see text.

2.4. Conclusions

Sample hydrolysis and storage conditions have been optimised to enable the quantification of isoflavones in clover leaves. Storage is best done at –20°C: for at least 1–2 weeks no significant loss of malonate esters is observed. Hydrolysis at elevated temperatures (83°C) should preferably be performed over a 2 h period without acidification. Under such conditions FGM and BGM are quantitatively converted into the corresponding glucosides while decomposition of the latter is still negligible. Hydrolysis at 83°C in the presence of acid provides a way to obtain additional information on the flavonoid distribution. In the present study, LC–APCI(-)-MS under conditions which are rather mild (pH 4.0) compared to those sometimes used in the literature proved a good means for analyte identification. Preliminary experiments using LC–FLU indicate the selectivity of this technique in flavonoid studies; it is currently the subject of further investigations. For quantification LC–UV was used for ten target compounds comprising aglycons, glucosides and glucoside malonates. For eight out of the ten target analytes, the concentrations in the clover leaves were found to range from 0.042 µg/g to 4.9 mg/g which opens the possibility to use these compounds as indicators to characterise wetland ecosystem properties.

Acknowledgement

We thank Ms. Antoinette Toebes at the Ecology and Ecotoxicology of Plants Department of the Free University for providing the clover samples.

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3 Natively fluorescent isoflavones exhibiting anomalous Stokes' shifts[1]

Summary

The fluorescence behaviour of nineteen flavonoids was studied. Three isoflavones, formononetin (F), ononin (FG) and daidzein (D) were found to exhibit large Stokes' shifts, possibly due to a change of the structure of the molecule from non-planar in the S_0, to planar in the S_1, state. Lifetime measurements were carried out using time-correlated single photon counting spectroscopy to further characterize the mechanism. These large shifts provide a high selectivity, so that fluorescent isoflavones can be readily detected in plant samples by means of reversed-phase liquid chromatography (LC) with fluorescence detection. Attention has to be paid to possibly fluorescent impurities in flavonoid standards, as was observed for daidzin (D), which is not fluorescent itself, but has a fluorescent isomer. To distinguish between the two compounds, LC with fluorescence and mass spectrometric detection was used to separate and identify the impurity.

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3.1 Introduction

Flavonoids are one of the most characteristic groups of compounds in higher plants, and are present not only in flowers and leafs but also in *e.g.* roots [1]. They are known for their UV-B protecting properties in plants, are used as food supplements and some of them, the isoflavonoids, are known to be phytoestrogens [2]. The ultimate objective of our present research programme is to use flavonoid profiles in wetland plants as chemical indicators for stress in wetland ecosystems. Obviously, this requires the use of various analytical methods, primarily gradient LC–UV, LC–MS and LC–MS/MS. In addition, it is of interest to find out whether fluorescence spectroscopy, known for its inherent selectivity, can be used as an alternative detection technique, either stand-alone or in combination with LC.

To the best of our knowledge, there is no report of a systematic and comprehensive study of the native fluorescence of flavonoids, though individual members of this class received much attention. This holds especially for 3-hydroxyflavone (3HF) and some of its derivatives. 3HF has been studied since the early 1980's [3,4] and is still receiving interest from various sides [5-7]. Its fluorescence behaviour is fascinating because it shows an extremely large wavelength difference between excitation and emission which is strongly dependent on solvent polarity, observations that have been interpreted by the ESIPT (excited state intra-molecular proton transfer) mechanism. After excitation, extremely fast (~30 fs) proton transfer takes place and emission of the tautomeric form is observed [7].

The present study deals with 19 representative flavonoids, *i.e.* flavones characterized by a chromone skeleton and a phenyl substituent at the C_2 position (3HF belongs to this group), isoflavones with the phenyl substituent at the C_3 position of the chromone group, and some flavanes in which the C_2–C_3 bond in the chromone skeleton is saturated. It will be shown that within this series of 19 flavonoids – apart from 3HF – only a few isoflavones show native fluorescence, characterized by very large Stokes' shifts that cannot be attributed to ESIPT.

3.2 Experimental

Materials

Daidzein, genistin, genistein, naringin, naringenin-7-glucoside, naringenin, formononetin and ononin were purchased from Roth (Karlsruhe, Germany), 3-hydroxyflavone, biochanin A, sissotrin and puerarin from Indofine Chemical Co. (Somerville, NJ, USA), rutin trihydrate and kaempferol from Fluka Chemie (Buchs, Switzerland), and quercetin, hesperidin,

hesperetin and ammonium formate from Sigma-Aldrich (Steinheim, Germany). Daidzin was obtained from both Roth and Indofine. Methanol and formic acid were purchased from J.T. Baker (Deventer, the Netherlands). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, the Netherlands). For the fluorescence experiments at different pH values, all solutions were prepared by diluting stock solutions in methanol with phosphate buffer, except for the pH 13 solution, to which extra 4 M NaOH was added to obtain the correct pH.

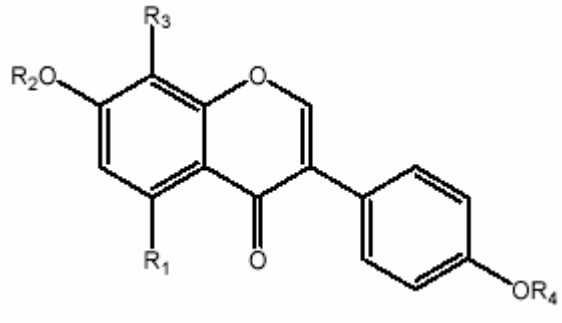
Instrumentation

Absorption spectra were recorded on a Varian Cary 50 Bio UV-Visible absorption spectrophotometer over the wavelength range of 200-500 nm. Fluorescence absorption and emission spectra were recorded on an LS-50-B spectrofluorimeter (Perkin Elmer, Beaconsfield, UK). LC–MS was performed on a Shimadzu (Princeton, NJ, USA) LC system, consisting of two LC-10A LC pumps, a DGU-14A degasser, a SIL-10AD auto-injector, a SCL-10A system controller unit and a SPD-10A UV detector (set at 265 and 290 nm), coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The mass spectrometer was provided with an electrospray interface. Mass spectra were acquired in the positive and negative ion mode in the *m/z* range of 150-650. The capillary temperature was maintained at 275ºC. MS/MS spectra were acquired by using collision-induced dissociation at 30% activation energy. The LC effluent was split, 0.2 ml/min being led to the mass spectrometer.

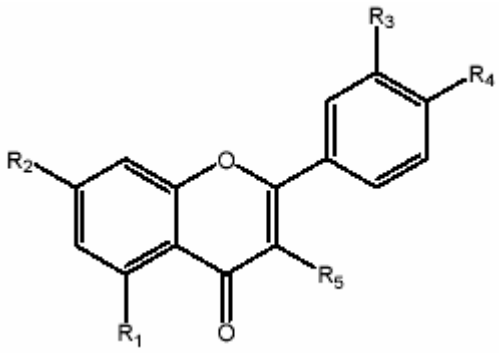
For LC–UV–FLU, LC was performed on a Hewlett-Packard (Palo Alto, CA, USA) Series 1090 LC system with a UV/DAD detector; fluorescence data were obtained with an Applied Biosystems (Foster City, CA, USA) Model 980 programmable fluorescence detector. Excitation was at 250 nm, and two emission filters were used; a 350 nm bandpass emission filter or a cut-off filter larger than 450 nm. A 250 x 4.6 mm I.D. 5 µm Zorbax SB-C_{18} column was used. The eluent was a mixture of methanol and aqueous 10 mM ammonium formate buffer, pH 4.0. The LC gradient used is shown in Table 1. All solvents were filtered and degassed with helium before use. The flow was 1.0 ml/min and the injection volume was 10 µl. The optimisation of the LC gradient and detection parameters in LC–UV–FLU and LC–MS was described in earlier work [8].

Lifetimes were measured using the time-correlated single photon counting technique [9]. The excitation source was a Coherent Mira 900 Ti-sapphire laser with a pulse width of about 3 ps. The output from the laser was frequency tripled to obtain an excitation wavelength of 250 nm. The energy was about 2 nJ/pulse, at a repetition rate of 76 MHz. Fluorescence was collected at a right angle from the sample cell and dispersed by a monochromator on a MPC-PMT (Hamamatsu R3809U-50) detector. Decay data were recorded with the help of an SPC-

630 (Becker-Hickl) module and analyzed using Fluofit software (Picoquant). For a good fit, χ^2 should be close to unity, and residuals should be distributed randomly [10]. The accuracy of the instrument was checked by recording the lifetimes of some standard compounds. The precision of the lifetimes was around 50 ps. The temperature was controlled and measured by a home-built system; the precision of the temperature measurements was ±1 K.



Isoflavones



Flavones, flavonols and flavanones

Isoflavones

Name	Acronym	R_1	R_2	R_3	R_4
Daidzein	D	H	H	H	H
Daidzin	DG	H	7-*O*-β-D-glc	H	H
Puerarin	PG	H	H	glc	H
Genistein	G	OH	H	H	H
Genistin	GG	OH	7-*O*-β-D-glc	H	H
Formononetin	F	H	H	H	CH_3
Ononin	FG	H	7-*O*-β-D-glc	H	CH_3
Biochanin A	B	OH	H	H	CH_3
Sissotrin	BG	OH	glc	H	CH_3

Flavones, flavonols and flavanones

Name	Acronym	R_1	R_2	R_3	R_4	R_5	C_2-C_3
Hesperidin	HG_2	OH	7-*O*-β-D-glcpyr	OH	OCH_3	H	single
Hesperetin	H	OH	OH	OH	OCH_3	H	single
Naringin	NG_2	OH	7-*O*-β-D-glcpyr	H	OH	H	single
Naringenin–7-glucoside	NG	OH	7-*O*-β-D-glc	H	OH	H	single
Naringenin	N	OH	OH	H	OH	H	single
Rutin trihydrate	RG_2	OH	OH	OH	OH	7-*O*-β-D-glcpyr	double
Kaempferol	K	OH	OH	OH	H	OH	double
3-Hydroxy-flavone	3HF	H	H	H	H	OH	double
Luteolin	L	OH	OH	OH	OH	H	double
Quercetin	Q	OH	OH	OH	OH	OH	double

Fig. 1. Structures and acronyms of the nineteen flavonoids studied. glc, glucoside; glcpyr, glucopyranosyl.

3.3. Results and discussion

Natively fluorescent flavonoids: pH effects

Out of the 19 flavonoids studied (see Fig. 1), only 3-hydroxyflavone and three of the isoflavones, ononin (FG), formononetin (F) and daidzein (D) showed native fluorescence in methanolic and aqueous solutions. Typical excitation and emission spectra are shown in Fig. 2; since the spectra of D are quite similar to those of F, they are not depicted separately. Fig. 2 shows that there is, for both F and FG – and the same holds for D – an exceptionally wide gap between excitation and emission, *i.e.* an exceptionally large Stokes' shift. Usually, such large shifts are related to excited-state deprotonation processes – as for instance in 3HF [3–7] – which will, of course, be pH-dependent. For this reason excitation and emission spectra were recorded over the pH range from 2 to 13. As can be seen in Fig. 2A, in the excitation spectrum of F an extra band at 340 nm starts to come up at a pH of about 6. Above pH 6, the pK_a value of F, the molecule is predominantly in its anionic form. Simultaneously with the change in the excitation spectrum, the maximum in the emission spectrum of F shifts to slightly shorter wavelengths, *i.e.* from 495 nm (the emission maximum of F) to 470 nm (the emission maximum of F^-). As is to be expected, no pH effect is observed for FG (Fig. 2B) because, due to the glucose group in the C_7 position, there are no ionisable groups.

FG as a model system

Since protonation/deprotonation effects apparently do not account for the Stokes' shifts in natively fluorescent isoflavones – very large Stokes' shifts are also observed for FG and F^- – other excited-state processes such as molecular rearrangements have to be considered. Since such processes might be influenced by solvent polarity, the proton donating/accepting character of the solvent or solvent viscosity, the fluorescence of FG was studied in various solvents. FG was used as the test compound since its fluorescence in an aqueous environment is not influenced by the pH, see Fig. 2B. The shape of the spectra which were obtained in protic solvents were found to be similar, apart from shifts in the wavelengths of the emission and excitation maxima. For instance, on going from methanol to ethanol, the emission maximum shifted substantially, *i.e.*, from 492 nm to 456 nm. The decrease of the Stokes' shift on going from methanol to ethanol is in line with the difference in polarity of these solvents, as expressed by their $E_T(30)$ values, a frequently used solvent polarity scale based on a single parameter [11,12]. For ethanol $E_T(30)$ is 51.9, significantly smaller than for methanol, which has an $E_T(30)$ of 55.4.

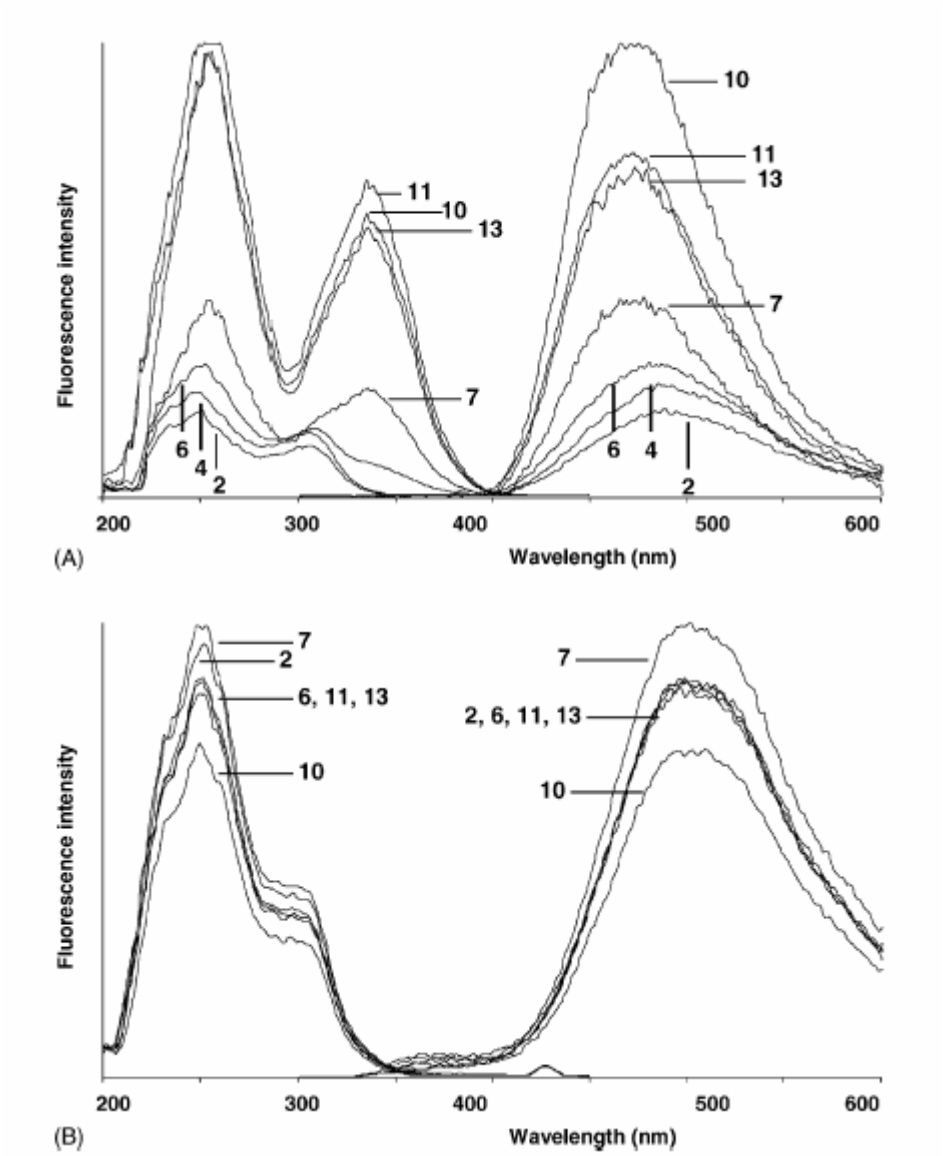


Fig. 2. Fluorescence excitation and emission spectra of (A) F and (B) FG in methanol:water (1:1, *v/v*) solutions at different pHs.

Contrary to the results obtained for protic solvents, in the aprotic solvents acetonitrile, dichloromethane and methylcyclohexane hardly any fluorescence was observed. Such a strong solvent influence is known from the literature if two close excited states of nπ* and ππ* character play a role, as for instance in 2-naphthylaldehyde [13]. However, a detailed theoretical treatment of the molecule at hand is beyond the scope of this paper. In fact, the weak fluorescence in acetonitrile could not be attributed to FG itself. The shapes of the associated excitation and emission spectra were completely different compared to those of FG in water (Fig. 3A); the emission maximum was found at the short wavelength of 350 nm and the corresponding excitation maximum at 230 nm. Presumably, in acetonitrile we are dealing with another fluorescent species, probably a decomposition product of FG. This is in line with

the fact that the absorption spectra in acetonitrile and water were also found to be distinctly different (Fig. 3B) and, furthermore, with the fact that the shapes of the excitation and absorption spectra in acetonitrile are not identical. Here it should be added that LC–FLU of FG did not reveal the presence of a fluorescent impurity. Since such decomposition in acetonitrile – which has not been reported in the literature – may well influence the results of conventional reversed-phase LC analyses, this phenomenon will be the subject of a more detailed study in the near future.

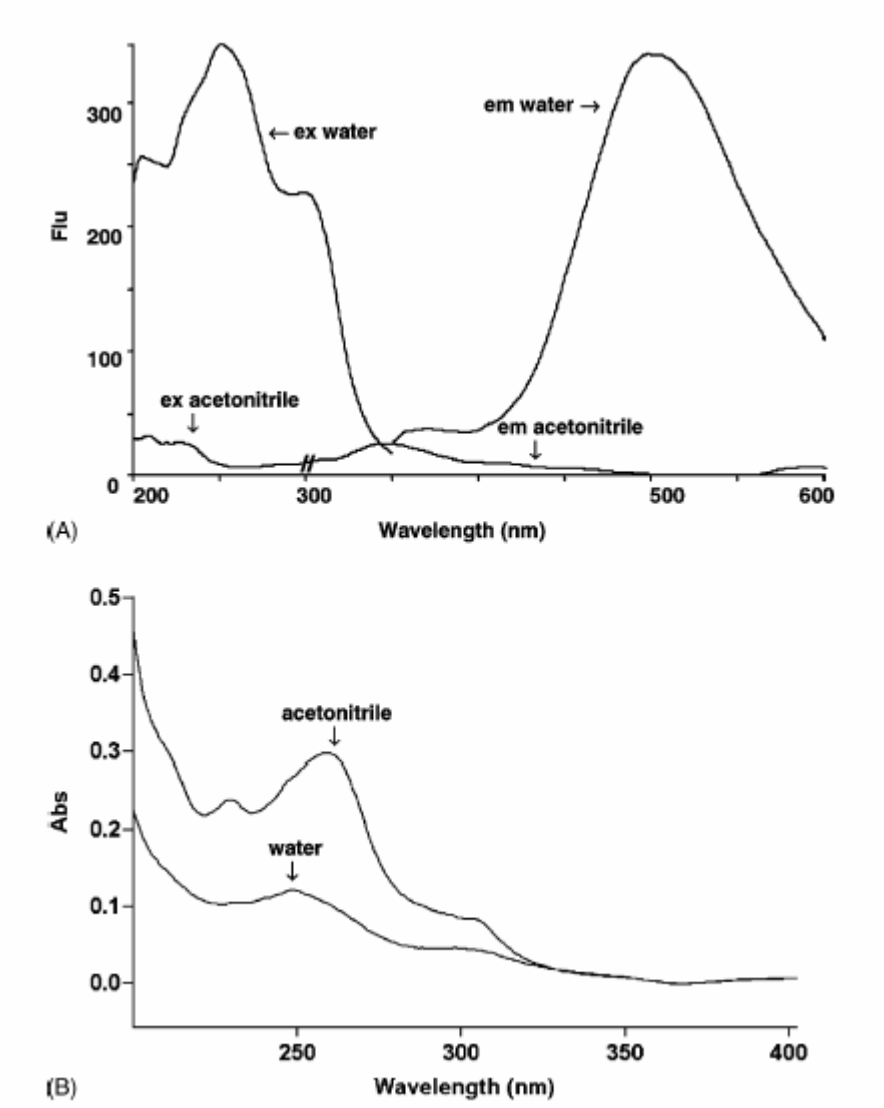


Fig. 3. (A) Fluorescence excitation (ex) and emission (em) spectra and (B) absorption spectra of FG in acetonitrile and in water (6 μM). Fluorescence ex and em slits were set at 15 nm.

The fluorescence of FG was also studied at low temperature. In methanol:ethanol (1:1, *v/v*) cooled to 77 K with liquid nitrogen, rather unexpectedly no fluorescence was observed at all, but in pure ethanol at 77 K, which forms a glassy matrix, FG did show very weak fluorescence, with the emission maximum at about 400 nm. Apparently, cooling not only causes an extreme reduction of the fluorescence intensity – a phenomenon quite different

from what is usually encountered in fluorescence studies where a temperature decrease often causes improved quantum yields – but also a large shift of the emission wavelength: from 456 nm to about 400 nm. One possibility is that the above temperature influence is related to the change in solvent viscosity. Therefore, the fluorescence spectra of FG were also recorded in ethylene glycol, a solvent with a viscosity of about 30 cP at room temperature, which is 30–50-fold higher than those of ethanol (1.07 cP) and methanol (0.63 cP), while its $E_T(30)$ value is 56.3, only slightly higher than that of methanol. If viscosity does not play a role at all, one would expect the emission maximum in ethylene glycol to be at a somewhat longer wavelength than that in methanol (493 nm). The emission maximum was found at 480 nm, *i.e.*, at a slightly shorter wavelength, which may indicate a viscosity effect, but the shift is too small to be conclusive. Apparently, also in ethylene glycol at room temperature the excited-state molecular rearrangement of FG is still too fast to allow its unambiguous establishment in steady-state experiments.

In view of the above results, time-resolved experiments should be performed to try to monitor such an excited-state molecular rearrangement. One may speculate that the molecule, after being excited to the S_1 state, has to undergo a structural rearrangement before being able to emit fluorescence, for instance from non-planar (the configuration in the S_0 state) to planar; intermediate states will emit at shorter wavelengths and with a lower fluorescence quantum yield. Fluorescence lifetimes of FG in methanol:ethanol (1:1, *v/v*) were measured at two emission wavelengths, *i.e.*, 440 nm and 480 nm, at room temperature and at 173 K; lower temperatures could not be handled in the time-correlated single photon counting experiment because of too strong a reduction in fluorescence yield. The wavelength of 480 nm reflects the red edge of the emission spectrum corresponding to the fully equilibrated excited-state molecules. The wavelength of 440 nm reflects the blue edge; if, as assumed, molecular rearrangement processes are operative, the emission at this wavelength may be partly due to not yet fully rearranged S_1-state molecules, for convenience denoted as FG[††]. The experimental results confirmed this interpretation: at room temperature the emission lifetimes at 440 nm and 480 nm were found to be the same, *i.e.* 3.2 (±0.1) ns. Apparently, under these conditions full S_1-state equilibration is achieved before emission takes place. At 173 K however, the red edge emission behaves significantly different from the blue edge emission (Fig. 4). The 480 nm curve was fitted with two mono-exponential functions, *i.e.*, a decay function representing a fluorescence lifetime of 16.4 (±0.5) ns (as expected, longer than the room temperature value of 3.2 ns) and also an in-growth function with a rise time of 1.5 (±0.1) ns. The latter function represents the proposed molecular rearrangement process: it takes about 1.5 ns before the molecules, after being excited, arrive in the planar excited state. To describe the 440 nm curve, two mono-exponential decay functions were needed, a rapid

one with a decay of 1.0 (±0.1) ns reflecting the further rearrangement of $FG^{\dagger\dagger}$ and a slow one of 10.5 (±0.5) ns, representing the fluorescence lifetime of $FG^{\dagger\dagger}$. The similarity of the rapid decay at 440 nm and the rise time at 480 nm points to an excited-state conformational change as assumed above. They are not identical because most probably we are dealing with a continuous relaxation process starting at the configuration of the initially excited state to the relaxed excited-state configuration. The spectra of $FG^{\dagger\dagger}$ and fully relaxed FG will partly overlap, both at 440 nm and 480 nm. We therefore conclude that the large Stokes' shifts observed for the fluorescent isoflavones, may be attributed to a structural change of the molecule from non-planar in the S_0, to planar in the S_1, state, as was previously found for the S_0 state of similarly structured compounds [14].

Daidzin impurity

One should be very careful in attributing a fluorescence emission to a particular flavonoid. In fact, a purity check, in which the LC–UV and the LC–FLU traces are compared, is crucial. Thus it was found earlier [8] that the fluorescence displayed by a methanolic solution of a daidzin (DG) standard from Roth had to be assigned to an unknown isomer of DG present in low concentration. In fact, DG itself does not show any fluorescence at all. This conclusion was confirmed by the observation that for a DG standard from another company (Indofine Chemical Co.) no fluorescent impurity was found. It should be noted that, apart from the fluorescence behaviour, DG and the isomer in the Roth standard have almost identical properties.

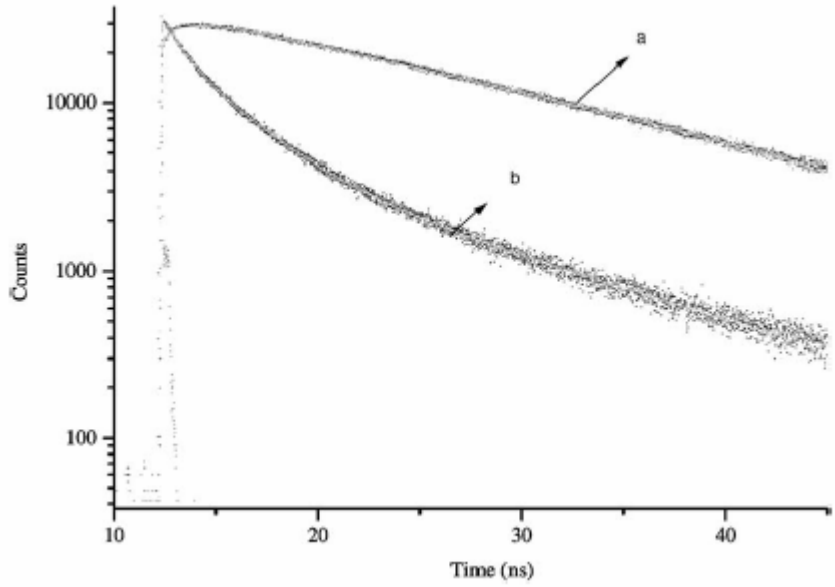


Fig. 4. Time-resolved emission of FG in methanol:water (1:1, *v/v*) at 173 K recorded at (a) 480 nm and (b) 440 nm.

When the methanolic solution of DG (Roth) was subjected to LC–ESI-MS in the positive mode, using the same LC conditions as above, two peaks were observed in the mass trace, *viz*. at 6.7 and 8.7 min. In the mass spectrum of the first peak, *m/z* 255 and 417 show up and have rather similar intensities, while in the mass spectrum of the second peak only *m/z* 417 is observed and *m/z* 255 is essentially absent; *m/z* 255 is formed after loss of a glucose group. According to the literature [15], when a glucose moiety is bound to oxygen, as is the case for DG, both $[M+H]^+$ and $[M+H–glc]^+$ ions will be observed in the full-scan mass spectrum, but with a C-bound glycoside, only $[M+H]^+$ will be observed. That is, the early eluting peak is possibly an O-bound glycoside and the second (fluorescent) peak, a C-bound glycoside. Puerarin, the C_8-bound glucoside isomer of DG, was also subjected to LC–FLU, but it did not show any fluorescence. To check whether both peaks in the DG standard contain a glucose group, ESI-MS/MS was performed with *m/z* 417 as precursor ion in the positive mode. The MS/MS spectra of both peaks contain mass 255, which is indeed the aglycon moiety. Nonetheless, the structure of the isomer is not yet completely established. To the best of our knowledge, the presence of a fluorescent isomer of DG has not been reported in the literature.

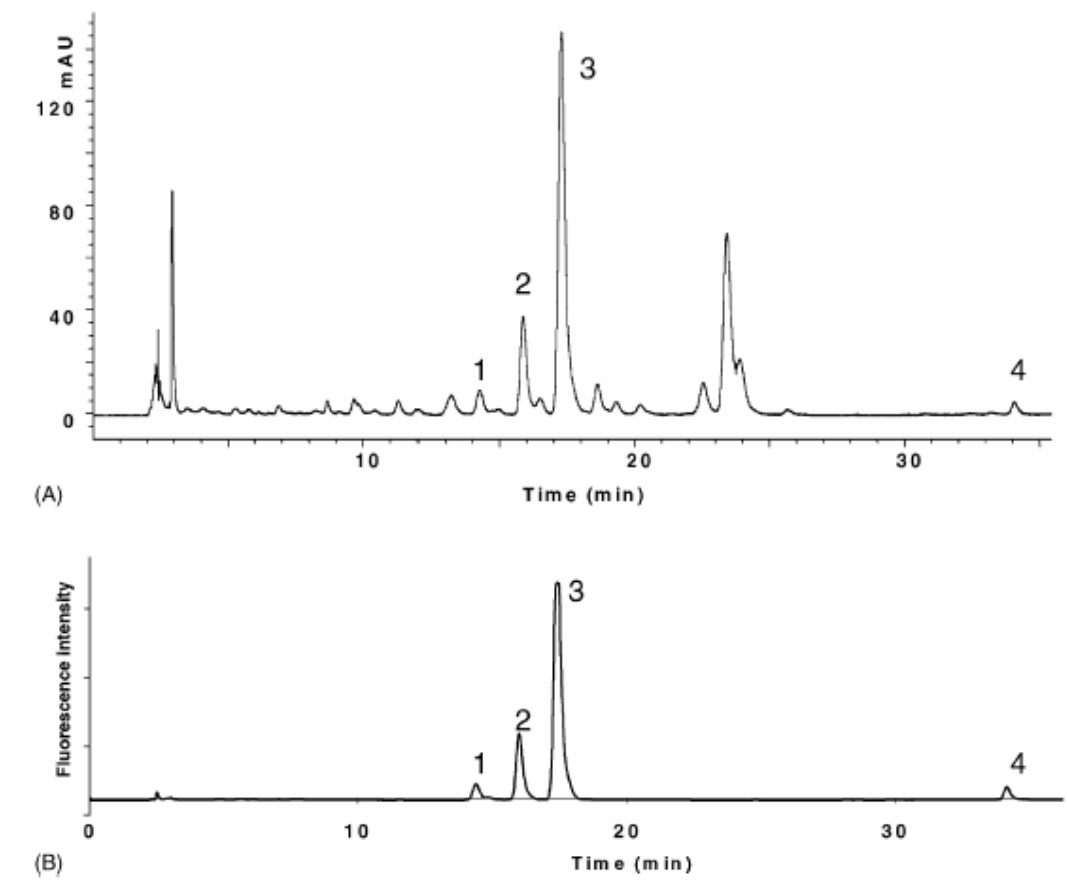


Fig. 5. (A) LC–UV_{265} and (B) LC–FLU trace of red clover extract. In (B) Ex at 250 nm, Em > 450 nm. For LC conditions, see Section 2.2. Peak assignments are given in Section 3.4.

LC–FLU analysis of real samples

Due to the large shifts between the excitation and emission wavelengths of the fluorescent isoflavones, high selectivity can be obtained in LC with fluorescence detection. This is particularly relevant if real samples have to be analyzed. This was denoted for wetland plant extracts, *viz.* for two clover extracts. In Fig. 5 the (expected) gain in selectivity upon going from LC–UV to LC–FLU is immediately apparent upon comparing Figs. 5A and 5B, which show the results for a red clover extract. When the fluorescence detector was equipped with a 350 nm bandpass emission filter (a wavelength at which isoflavones hardly show fluorescence) no peaks were observed above 10 min (results not shown). If, instead, a cut-off filter of >450 nm is applied (Fig. 5B), four prominent peaks show up at retention times between 13 and 35 min. These can be attributed to the fluorescent isoflavones, [3] formononetin–7-*O*-β-D-glucoside–6"-*O*-malonate (FGM), [1] an isomer of FGM, [2] FG and [4] F. Closely similar results were obtained with a white clover extract, for which no results are shown here. Limits of detection (LODs, *S/N*=3) were determined for FG and F on both detectors; for the UV detector LODs were 1 and 0.5 mg/l, and for the fluorescence detector 0.1 and 0.05 mg/l, respectively. Repeatability of the method was satisfactory; RSD values of the peak area of the 10 major peaks in the chromatogram were 1-5% (n=7).

3.4. Conclusions

Large Stokes' shifts for fluorescent isoflavones are possibly due to a change of the structure of the molecule from non-planar in the S_0, to planar in the S_1, state. This phenomenon deserves further spectroscopic study. From an analytical point of view these large shifts provide a high selectivity, so that fluorescent isoflavones can be readily detected in real samples by means of reversed-phase LC with fluorescence detection, applying methanol/water gradients and a large wavelength difference between λ_{ex} and λ_{em}. Because of instability problems of at least some of the target analytes, acetonitrile should be avoided as a solvent.

3.5 References

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4. Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments[1]

Summary

With fifteen flavonoids as test compounds, the analytical performance of four modes of LC–MS, MS^n and tandem MS operation (APCI, ESI; positive and negative ionization) was compared for two mass spectrometers, a triple-quadrupole and an ion-trap instrument. Two organic modifiers, methanol and acetonitrile, and two buffers, ammonium acetate and ammonium formate, were used. In general, the use of APCI in the negative ion mode gave the best response, with the signal intensities and the mass-spectral characteristics not differing significantly between the two instruments. The best results were obtained when methanol–ammonium formate (pH 4.0) was used as LC eluent.

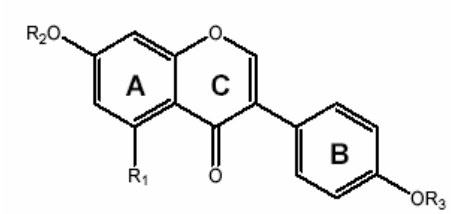
Under optimum conditions full-scan LODs of 0.1-30 mg/l were achieved in the negative APCI mode. Here it needs to be emphasized that up to 2-order response differences were found both between analytes and between modes of ionization. This implies that one should be very cautious when interpreting data on the screening of real-life samples. The main fragmentations observed in the MS^n spectra on the ion-trap, or the tandem MS spectra on the triple-quadrupole were generally the same. The advantage of the former approach is the added possibility to ascertain precursor → product ion relationships.

[1] E. de Rijke, H. Zappey, F. Ariese, C. Gooijer, U.A.Th. Brinkman, *J. Chromatogr. A* 984 (2003) 45.

4.1 Introduction

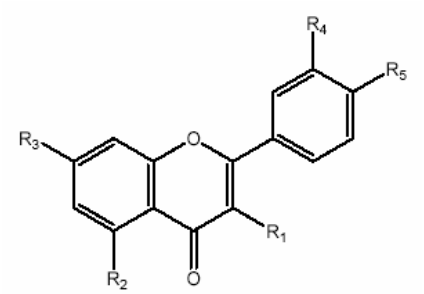
In the literature, various groups of workers have devoted considerable attention to the determination of flavonoids in a variety of samples [1–12]. Flavonoids, which are one of the most characteristic classes of compounds in higher plants, comprise a wide group of structurally related compounds with a chromane skeleton and a phenyl substituent in the C2 (flavones) or C3 (isoflavones) position. Structures and abbreviations used in this paper are given in Fig. 1.

Isoflavones



Name	Abbrev.	R_1	R_2	R_3	MW
Daidzein	D	H	H	H	254
Daidzin	DG	H	7-*O*-β-D-Gly	H	416
Genistein	G	OH	H	H	270
Genistin	GG	OH	7-*O*-β-D-Gly	H	432
Formononetin	F	H	H	CH_3	268
Ononin	FG	H	7-*O*-β-D-Gly	CH_3	430
Biochanin A	B	OH	H	CH_3	284
Sissotrin	BG	OH	7-*O*-β-D-Gly	CH_3	446

Flavones



Name	Abbrev.	R_1	R_2	R_3	R_4	R_5	C_2-C_3	MW
Hesperidin	HG2	H	OH	7-*O*-β-D-Neohesp	OH	OCH_3	single	610
Hesperetin	H	H	OH	OH	OH	OCH_3	single	302
Naringin	NG2	H	OH	7-*O*-β-D- Neohesp	H	OH	single	580
Naringenin-7-glucoside	NG	H	H	7-*O*-β-D-Glu	H	OH	single	434
Naringenin	N	H	OH	OH	H	OH	single	272
Rutin trihydrate	RG2	3-*O*-β-D-rutinoside	OH	OH	OH	OH	double	610
Kaempferol	K	OH	OH	OH	OH	H	double	286

Fig. 1. Structures of the flavonoids studied and their acronyms; Glu, glucoside; Neohesp, neohesperidose. Rings and bonds indicated by A, B, C and 1-4, respectively, are discussed in Section 3.5.

Flavonoids that have a single C2—C3 bond are called flavanones; with an OH group in the C3 position they are called flavonols. Flavonoids are usually found in plants as glycosides, *i.e.* provided with one or more sugar substituents such as galactose, rhamnose or glucose, or glucoside malonate. Because flavonoids are moderately polar, thermolabile compounds, they are best analyzed with reversed-phase LC. Obviously — especially if identification of individual flavonoids is important — advanced hyphenated techniques have to be used, *e.g.*, LC–MS or LC–MS/MS. Various ionization methods, such as thermospray, fast atom bombardment and atmospheric pressure ionization (API) have been applied to determine flavonoids. Today, API-MS methods in particular receive much attention. For the broad class of flavonoids, which comprises aglycons, glycosides and conjugates such as malonates and acetates, both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are used. Good results can be obtained with both techniques, even with ESI, although the flavonoids — especially the aglycons — are not very polar. Selected papers on the determination of flavonoids with LC coupled to positive- or negative-mode APCI and/or ESI are listed in Table 1.

Table 1. Selected papers on LC–API-MS(/MS) of flavonoids*

Analytes	Sample	LC eluent	API mode	Type of MS	Ref.
Flavonoids	Fresh herbs	MeOH-water, 1% FA	APCI–	Q	1
Isoflavones	Soy foods	AcN-water, 0.1% TFA or 0.1% AA	APCI ± and IS ±	QqQ	2
Flavonoids	Red clover	AcN-water, 0.25% AA	ESI ±	Q	3
Phenolic compounds	Soy, onions	AcN-water, 10%FA	ESI–	Q	4
Phenolic compounds	Olives	MeOH-water, 1% AA	ESI ±	QqQ	5
C-Glycosidic flavonoids	Not applied	AcN-water, 0.5% AA	APCI ± and ESI ±	IT and Q-TOF	6
Flavonoids	Wood pulp, waste water	MeOH-water, 0.5% AA	ESI ±	QqQ	7
Flavonoids	Urine	MeOH-AcN- water, 0.5% FA	APCI–	Q	8
Flavonoid aglycones	Not applied	MeOH-water, 0.1% FA	ESI–	IT	9
Flavonoids	Onion, blossom and St. John's Worth	AcN-water, 20 mM TFA	ESI+	IT	10
Flavonoids	Not applied	MeOH/AcN-water, 0.1-0.4% FA or 10 mM AAc or 0.1% AH, 0.05% TFA	IS ±, APPI ± and APCI ±	QqQ	11
Isoflavones	Red clover	AcN-water, 0.2%AA	ESI+	Q	12

* MeOH: methanol, AcN: acetonitrile, FA: formic acid, AA: acetic acid, TFA: trifluoroacetic acid, AAc: ammonium acetate, AH: ammonium hydroxide, Q: single quadrupole, QqQ: triple quadrupole, IT: ion trap, TOF: time of flight, IS: ion spray, APPI: atmospheric pressure photoionisation.

Some authors have stated that there are differences in analyte response between the different ionization modes [5], and that the choice of organic modifier (and buffering agent) may also play a role, but so far, only Rauha *et al.* [11] recently studied solvent effects on the ionization and detector sensitivity of five flavonoids. However, generally speaking little systematic work has been performed in this area. Since the goal of most studies, including ours, is to monitor target analytes as well as to screen for unknown flavonoids in plants or food, the analyte responses obtained with the various methods play an important role and require due attention.

The aim of the present paper is to compare the analytical performance of the APCI and ESI API techniques, both in the positive and negative mode. This was done for two different mass spectrometers, a triple-quadrupole and an ion-trap instrument, since their differences in design may affect the MS mode selection. Standard solutions containing fifteen selected flavonoids were used to study the differences in analytical performance.

4.2 Experimental

Materials

Daidzin, daidzein, genistin, genistein, naringin, naringenin-7-glycoside, naringenin, formononetin and ononin were purchased from Roth (Karlsruhe, Germany), biochanin A and sissotrin from Indofine Chemical Co. (Somerville, NJ, USA), rutin trihydrate and kaempferol from Fluka Chemie (Buchs, Switzerland), and hesperidin, hesperetin, ammonium acetate and ammonium formate from Sigma-Aldrich (Steinheim, Germany). The flavonoids were analyzed in two mixtures (see Section 3.1); their concentration was 20 mg/l in methanol.

Methanol and formic acid were from J.T. Baker (Deventer, the Netherlands), and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, the Netherlands).

Methods

Analyses were performed on two LC–MS systems. One was a Shimadzu (Princeton, NJ, USA) LC system, consisting of two LC-10A LC pumps, a DGU-14A degasser, a SIL-10AD auto-injector, a SCL-10A system controller unit and a SPD-10A UV detector (set at 250, 265 and 290 nm), coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion-trap mass spectrometer. The other LC–MS consisted of a HP 1090 Series II LC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a Quattro II triple-stage quadrupole MS (Micromass, Altrincham, UK). Both mass spectrometers had APCI and ESI interfaces. A 250 x 4.6 mm I.D. 5 μm Zorbax SB-C_{18} column was used for separation on both systems. The LC

eluent consisted of a mixture of an organic modifier and an aqueous volatile buffer. Four combinations were tested: either 10 mM aqueous ammonium formate (AF) or 10 mM aqueous ammonium acetate (AA) buffer (both pH 4.0), and methanol (MeOH) or acetonitrile (AcN). The AF and AA buffer pHs were adjusted by adding formic acid and acetic acid, respectively. The LC gradients for the two flavonoid mixtures are shown in Table 2. The flow was 1.0 ml/min and the injection volume, 10 µl. All solvents were filtered and degassed with helium before use.

APCI and ESI mass spectra were acquired in the positive ion (PI) and negative ion (NI) mode in the range of *m/z* 150-650. On the ion-trap MS, the capillary temperature for APCI was maintained at 250ºC and the vaporizer temperature at 450ºC. These intermediate values were used because varying the capillary and vaporizer temperatures within the range specified by the manufacturers did not noticeably change the mass spectra. For ESI, the capillary temperature was optimized by direct infusion of a 5-mg/l methanolic solution of genistin while varying the temperature from 200ºC to 340ºC. The total signal in full-scan acquisition did not vary much with temperature, but at temperatures below 270ºC increasing adduct formation (Na^+ adducts and double ions in the PI mode and formic acid adducts in the NI mode) occurred. Therefore, the capillary temperature was set at 275ºC. After optimization for all flavonoids, the capillary voltage was set at 4 V for PI ESI, –4 V for NI ESI, 46 V for PI APCI and –12 V for NI APCI. The APCI corona discharge current was 10 µA. The sheath-gas (nitrogen) flow rates for APCI and ESI were 80 and 40 U, respectively, which corresponds to approximately 200 and 20 liters/hour, and the auxillary gas (nitrogen) flow rate was 20 U for both APCI and ESI. For MS^n spectra of selected precursor ions, the activation energy was optimized between 20% and 50% in the PI and NI ESI mode by direct infusion of 5-mg/l methanolic solutions of all compounds. For all compounds, the highest total signal in full-scan acquisition was obtained at 30%. For MS^n experiments, higher activation energies were necessary to obtain complete fragmentation (see Section 3.5).

Optimization of the source temperature and cone voltage settings for the triple-quadrupole MS was performed in the same way as for the ion-trap instrument. The source and probe temperatures for APCI were maintained at intermediate values; 150ºC and 400ºC, respectively. For ESI the source temperature was varied between 70ºC and 140ºC; for temperatures below 80ºC more adduct formation occurred. Therefore, the source temperature used for all analyses was 120ºC for ESI. After optimization for all flavonoids, the cone voltage for ESI and APCI was set at –50 V (NI) or 50 V (PI), the probe voltage was set at 3.5 V for PI ESI and –3.0 V for NI ESI. The APCI corona discharge was 5 kV. Sheath-gas (nitrogen) flow was 150 liters/hour for APCI and nebulizing gas (nitrogen) was 15 liters/hour for ESI. Drying gas (nitrogen) flow was 200 liters/hour for ESI and 300 liters/hour for APCI.

With both instruments, for APCI the LC flow was directed into the mass spectrometer without stream splitting, while for ESI the LC flow was split and 0.2 ml/min was sent to the mass spectrometer.

4.3 Results and Discussion

LC–UV

In order to obtain a complete separation of the fifteen selected flavonoids, long gradient LC runs would be needed, since the flavonoid glycosides elute at closely similar retention times. Therefore, the fifteen compounds were divided into two groups, which of course doubled the number of runs, but shortened the total time of analysis. Separation of the 8-compound mixture was achieved under the gradient conditions given in Table 2 A and B. For the 7-compound mixture a slightly different gradient had to be used (Table 2 C and D).

Table 2. Gradient conditions (vol. %) for eight-compound mixture (A and B) and seven-compound mixture (C and D) with MeOH or AcN, and AF or AA, both at pH 4.0.

	Time (min)	% MeOH or % AcN	% AF or AA
A	0	30	70
	2	40	60
	10-12	55	45
	19-28	80	20
	31	30	70
B	4	30	70
	9-14	45	55
	17	55	45
	22-25	60	40
	30	30	70
C	0	20	80
	20	60	40
	22-24	95	5
	29	20	70
D	0- 3	20	80
	10-13	30	70
	20	40	60
	25	20	80

In Fig. 2 the UV responses of the fifteen flavonoids are shown for the four buffer/modifier combinations tested. The response is expressed as peak area, recorded at 250 (FG, F, DG, D), 265 (BG, K, B, GG, RG2, G) or 290 nm (NG2, HG2, H, NG, N), depending on the absorption maximum of each compound. All flavonoids can be detected with all buffer/modifier combinations tested, but it depends on the compound which combination is

best. For most flavonoids, the highest peak areas were obtained with MeOH/AF, but G, GG, NG and RG2 gave up to 50% higher responses when using AcN as a modifier. For K and DG, the responses were relatively low when using AcN/AF. The choice of the buffer, AF or AA, was found to be most important for F. To the best of our knowledge, such phenomena have not been reported and/or discussed in the literature. It has to be noted that we found that FG is not stable in pure AcN solution; a few minutes after dissolution a degradation product is formed which can be monitored in the absorption spectrum [13]. Therefore, elution with AcN may be thought also to have an effect on the degradation of other flavonoids. However, for FG in the AcN/buffer mixtures applied no significant effects were observed. Apparently, the presence of water slows down the degradation process.

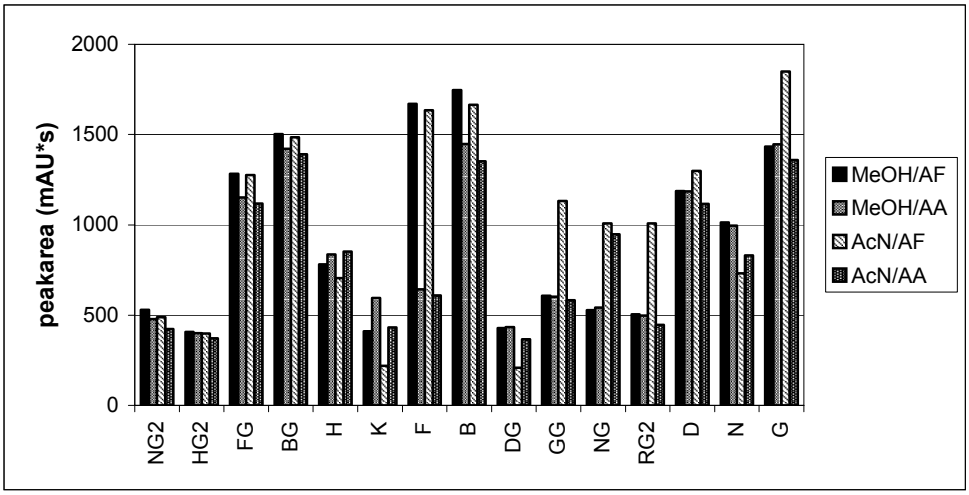


Fig. 2. Effect of the LC-eluent composition on the UV signal of 15 flavonoids expressed as peak areas at 250 (for FG, F, DG, D), 265 nm (for BG, K, B, GG, RG2, G) or 290 nm (for NG2, HG2, H, NG, N). AF and AA: pH 4.0.

In Table 3 the retention times of the flavonoids are given, with MeOH/AF or AcN/AF as LC eluent. Using AA as a buffer instead of AF has no influence on the retention times. With the MeOH-based gradient, the retention times of all compounds were longer than with AcN. With the 8-compound mixture, the differences were 1.4-3.4 min, and with the 7-compound mixture, 2.6-8.0 min. Other than that, the behaviour of the analytes was essentially the same with both modifiers; the only exceptions were: a change of elution order of K and H upon exchanging MeOH for AcN and the rather rapid elution of RG2 when the latter solvent was used. Scrutiny of the literature did not reveal any paper that reports such changes of elution order upon exchanging MeOH for AcN. In all LC runs, the peaks of the glycosides were somewhat sharper when using AcN as a modifier instead of MeOH.

Table 3. Retention times (min) of (A) 8-compound mixture and (B) 7-compound mixture with two different LC gradients.

	Compound	Retention time (min)	
		MeOH–AF	AcN–AF
A	NG2	7.3	5.9
	HG2	7.7	6.1
	FG	9.9	8.0
	BG	13.8	10.7
	H	14.1	12.5
	K	15.0	12.1
	F	17.8	14.3
	B	19.5	18.3
B	DG	7.9	3.7
	GG	10.7	6.5
	NG	12.3	8.9
	RG2	12.5	4.5
	D	18.3	12.1
	N	20.2	17.6
	G	21.2	17.5

The influence of the buffer pH on the UV signal was studied with the MeOH/AF gradient as an example. The pH of the AF buffer was varied between 2.8 and 4.7. Changing the pH of the buffer had hardly any effect on the UV signal intensity (not shown); for all compounds it was constant within ± 2%. Calibration plots were constructed to determine the analytical performance of the LC–UV method by injecting a dilution series of the two standard mixtures containing 0.16–20 mg/l of all flavonoids (8 data points in duplicate). R^2 values were between 0.992 and 0.999 in all cases. For all flavonoids, LODs (S/N=3) were found to be 0.01-0.16 mg/l.

One may conclude that for LC–UV, the MeOH/AF (pH 4.0) combination is to be preferred. However, in several cases AcN/AF also yields good results. Furthermore, in-line recording of the UV response was an appropriate tool to monitor the quality of the LC runs in the subsequent LC–MS studies.

LC–MS

Because our goal was to develop a method compatible with LC–UV *and* LC–MS, we did not yet discard any of the four eluent combinations, especially not because the buffer pH was expected to play an important role in LC–MS. In all cases, a UV detector was used in-line to verify the undisturbed operation of the system. Peak areas of the fifteen flavonoids in the LC–MS full-scan chromatograms were compared for the four buffer/modifier combinations. Data were recorded on the quadrupole and the ion-trap MS in both the PI and NI, and ESI and APCI modes. The results obtained on the two instruments were similar. The highest responses were obtained in the NI modes. For all flavonoids, the differences in response observed for the ionization modes were very large (up to several orders of magnitudes), much larger than

the differences observed for the eluent combinations (up to 5-fold). In choosing the best ionization mode, the main criterion was the detectability of as many compounds as possible in the mixtures. We also considered a high signal in combination with a low background. For all ionization modes considered, the only exception being NI APCI, one or more flavonoids – especially the glycosides – could not be detected with any of the eluent combinations tested (data not shown) and in both positive modes the background signal was high. In both APCI modes MeOH/AF was the best eluent combination, in the ESI modes for some flavonoids MeOH/AF was best and for others MeOH/AA. In view of the above-mentioned criteria, we focussed our attention on NI APCI. An example is shown in Fig. 3; these results were obtained on the quadrupole instrument in the full-scan NI APCI mode. All flavonoids except RG2 were detected with all four eluent combinations (200 ng injected). The repeatability of the results was satisfactory.

As regards the differences in detectability, one example is that the glycosides have a much lower response than the aglycons. With reference to Fig. 1, one can see that if R_2=H for the isoflavones or R_3=OH for the flavones, the intensity is much higher than if R_2=Gly for the isoflavones or R_3=OGly for the flavones. These differences, which cannot be explained by the less than two-fold differences in molar concentrations between the glycosides and corresponding aglycons, are apparently related to the nature of the phenolic OH groups. An aglycon with a free OH group is more easily dissociated into a stable anion than a glycoside. Another explanation may be that glycosides have a higher vaporization temperature so that they cannot be vaporized as efficiently as aglycones.

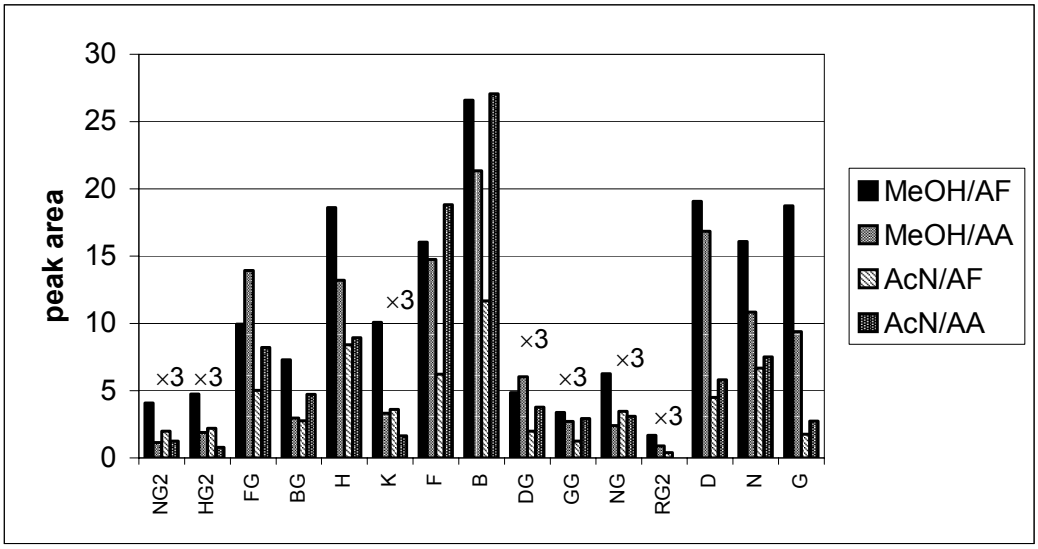


Fig. 3. Effect of LC-eluent composition on the NI APCI full-scan MS signal of 15 flavonoids. pH of both AF and AA, 4.0. For LC conditions, see Section 4.2.

For most flavonoids (11 out of 15), MeOH/AF was found to give the best results, although for several compounds either MeOH/AA or AcN/AA gave similar responses. The good results obtained with AF as buffer may be explained by the stronger acidity of formic acid compared to acetic acid. Both gas-phase and liquid-phase phase acidities may play a role; also the gas phase acidity of formic acid seems to be higher than that of acetic acid [14]. The higher anion concentration of the former acid at pH 4.0 will cause the ionization efficiency to be higher also. Unexpectedly, the use of AcN/AF often led to 2-3-fold lower peak areas; apparently, the ionization efficiency is much lower.

Using the MeOH/AF gradient, the influence of the buffer pH on the MS signal intensity of the 15 compounds was studied in the 2.8-4.7 range. Fig. 4 shows that for most compounds the extracted-ion MS signal ($[M–H]^-$) is highest for pH 3.8-4.3. Proton abstraction from phenolic OH groups to form $[M–H]^-$ anions should be more efficient at higher pH values and a similar mechanism is presumably operative in the gas phase. This trend is indeed observed but the highest pH value studied, pH 4.7, does not give the highest ionization efficiency. It is interesting to note that this phenomenon has been reported before for ion-spray (IS) and has been related to a progressively less efficient ion evaporation of anions at higher pH values [11]. In Fig. 4 extracted-ion data were used because in the full-scan MS chromatograms recorded at lower pHs, the peaks of some of the analytes could hardly be observed against the background noise. This noise is probably caused by the complexation of contaminants present in the eluent with the excess of hydrogen ions. More importantly for the present study, the relative intensities of the extracted-ion and full-scan data were found to be similar at the several pH values tested. For the rest, within the pH range tested, an optimum was reached at pH=4.0, which was used for all further work.

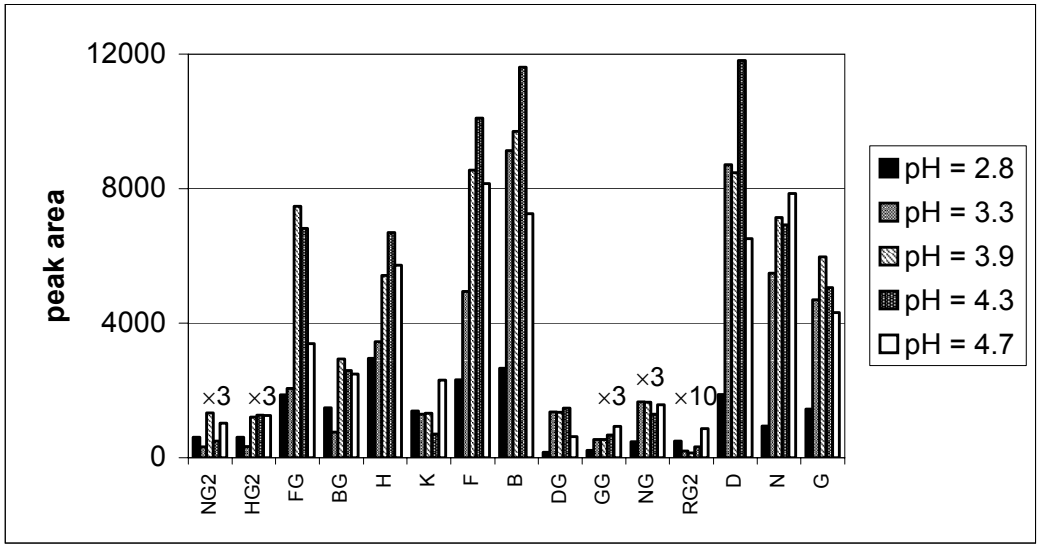


Fig. 4. Effect of AF buffer pH (MeOH used as modifier) on the extracted ion quadrupole NI APCI MS signals of $[M–H]^-$ of 15 flavonoids. For LC conditions, see Section 4.2.

Limits of detection (LODs, S/N=3) were determined on both instruments from full-scan chromatograms recorded using the MeOH/AF eluent combination (Table 4). On both machines, the LODs are lower in the NI than in the PI mode for both ESI and APCI, primarily because of the stronger background noise that is observed in the PI mode. The median values are in the 1-5 mg/l range. Generally speaking there is not too much difference between the LODs calculated for the two instruments irrespective of the ionization mode used, and even some of the PI-mode operations may come in useful in specific situations (also see below). Although no detailed study was made of the detectability enhancement that can be achieved via the use of extracted-ion traces, the general experience gained when constructing Fig. 4 was that a 2-3-order improvement, *viz*. to low-µg/l concentrations, was generally obtained.

Table 4. Ranges and median values of full-scan LODs (mg/l) of 15 flavonoids in LC–MS*

Instrument	ESI		APCI	
	NI	PI	NI	PI
Ion-trap	0.1- 4 (1)	0.1-20 (5)	0.1-30 (3)	2-40 (14)
Quadrupole	0.2-30 (5)	1-50 (15)	0.2-30 (3)	0.4-30 (8)

*MeOH/AF eluent; for details, see text.

Ionization mode selection

As a more detailed presentation of the general observations summarized above, Fig. 5 shows the S/N ratios of all flavonoids on both instruments and for all four ionization modes. The preferred MeOH/AF (pH 4.0) gradient was used in all instances. As was already indicated above, in almost all cases, the responses are best in the NI mode, with APCI overall in first, and ESI in second place. However, some additional conclusions can be drawn. On the quadrupole MS, PI APCI also seems to be a good choice for the eight isoflavones, but not for the other flavonoids. On the ion-trap MS, however, the results obtained with PI APCI are very poor for all analytes, while PI ESI yields satisfactory results in several cases (specifically D and DG). We have no explanation for this behaviour and, to the best of our knowledge, the published literature does not provide any clues either. In conclusion, a single LC–MS run using NI APCI will suffice to obtain good results for all test analytes but RG2, on either instrument. If, for increased confidence, an additional run is preferred, NI ESI can be performed.

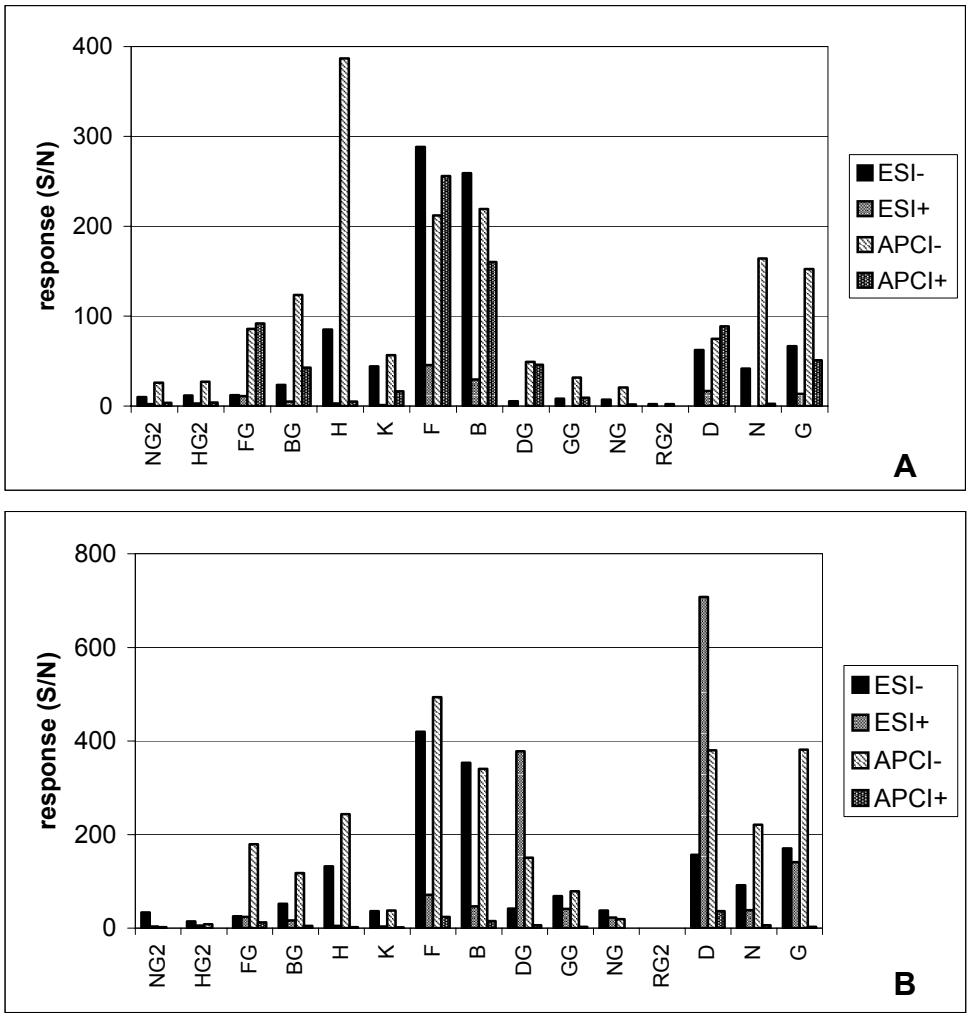


Fig. 5. Responses (S/N) of 15 flavonoids with different ionization modes in full-scan MS on (A) quadrupole and (B) ion-trap instrument. LC eluent: MeOH/AF (pH=4.0).

Finally, Fig. 6 shows LC–MS traces of the 8-compound mixture with APCI (A) and the 7-compound mixture with ESI (B), in both cases using PI and NI. One observation that can now be made more easily than before is that the NI *vs*. PI differences are much more dramatic when ESI is used. With NI, stable phenolate anions are formed. In PI, the noise level is relatively high and, under the conditions used, some peaks actually disappear in the background. A possible explanation for the low signal intensity is that the flavonoids, which do not contain nitrogen atoms, have a low basicity in the liquid phase and therefore the cation formation will be low for PI ESI.

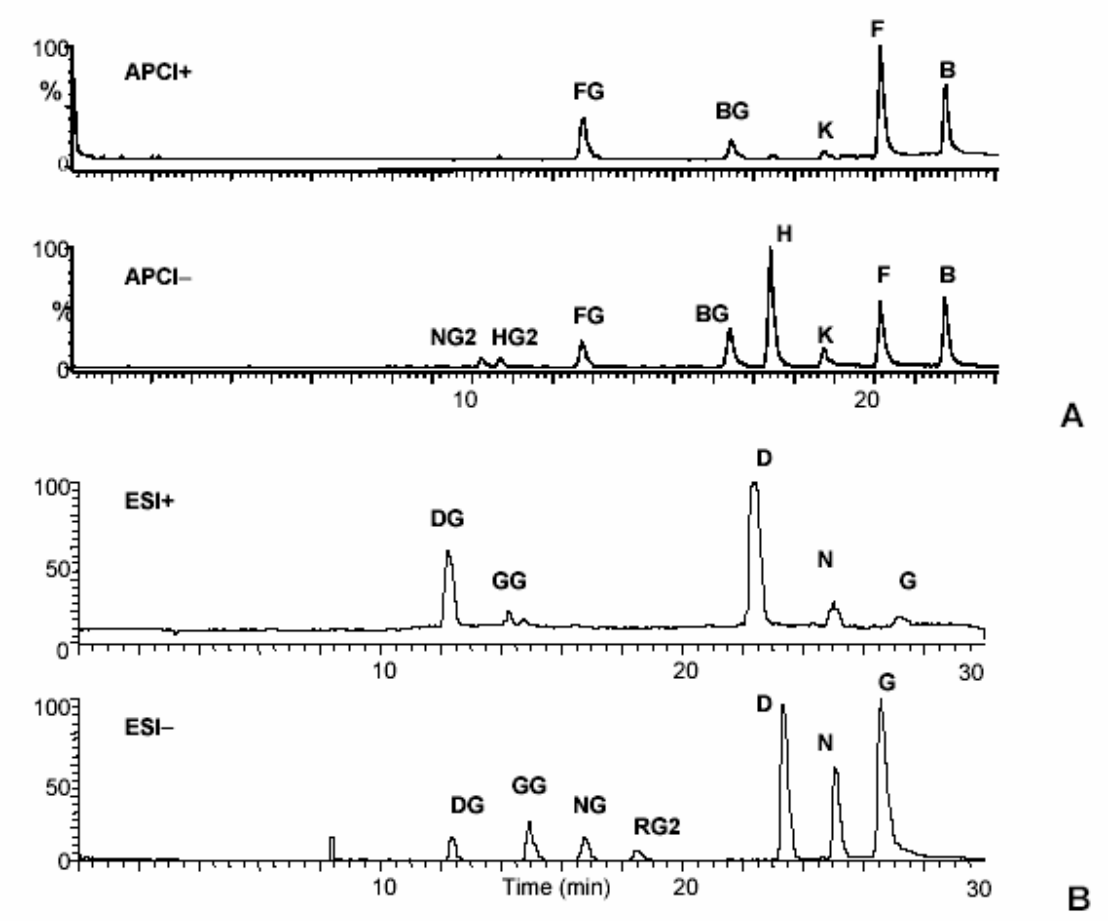


Fig. 6. Full-scan LC–MS of (A) 8-compound mixture using PI and NI APCI on quadrupole instrument and (B) 7-compound mixture in PI and NI ESI on ion-trap instrument. LC eluent: MeOH/AF (pH=4.0).

Analyte behaviour under various MS conditions

The main ions that showed up in the mass spectra of the flavonoids were: the pseudo-molecular ions, $[M–H]^-$ or $[M+H]^+$, a formic acid adduct, $[M+45]^-$, and – when the analyte was a glycoside – $[M–Gly]^-$ or $[M–2Gly]^-$, caused by loss of glycose, which agrees with literature data [1,5-7,9,10]. For the diglycosides, fragments formed by the loss of a single glycose moiety were not observed. Table 5 summarizes the results obtained for all four ionization modes, while grouping the analytes into flavonoids with two, one or no glycose moieties. Three ranges of relative abundances were used, 50–100%, 10-50% and 0–10%. The three most striking conclusions that can be drawn from the table are: (i) formic acid adduct formation occurs in the NI modes only; (ii) there is more fragmentation in ESI than in APCI, but only in the PI mode; (iii) generally speaking, the mass spectra on the ion-trap and the quadrupole instruments are closely similar.

As regards formic acid adducts (for the glycosides only), in NI APCI and NI ESI the adduct formation of an available anion, formate, with a neutral flavonoid molecule successfully competes with proton abstraction from the weakly acidic flavonoids by an

available base to form an $[M–H]^-$ anion. This is demonstrated by the relatively high abundance of $[M+45]^-$ ions. In PI, on the other hand, protonation of flavonoids by a suitable proton donor is a facile and a thermodynamically favorable process. Consequently, adduct formation to form stable $[M+47]^+$ ions is not favoured.

As regards the mass spectra, there generally is little difference between the ESI and APCI mass spectra in PI, while in NI more fragments are formed in ESI. Apparently, in the latter instance more in-source fragmentation takes place. In PI, stable protonated ions are formed which do not show extensive fragmentation, while in NI less stable adduct ions are formed that are more prone to fragmentation. In addition, one should consider that different probes are used for ESI and APCI. That is, gas flows and kinetic energies are somewhat different, which can affect the fragmentation.

One somewhat unexpected observation is the difference in fragmentation for flavonoids having two or one glycose moiety: only the latter class shows appreciable aglycon formation, even though the charge is on the flavonoid part of the molecule and the bond strength between that part and the single or double glycose moiety is the same.

Thirdly, irrespective of the ionization mode selected, the mass spectra acquired on the ion-trap and the quadrupole instrument were highly similar. This close similarity allowed us to summarize the results from both instruments in a single set of data (Table 5). The only exception was that, for some flavonoids, more formic acid adducts were formed on the ion-trap MS. The most striking example is NI APCI MS of DG (daidzin; one glycose moiety). On the ion-trap instrument, the $[M–Gly]^-/[M+45]^-$ ratio was 1:1 and $[M–H]^-$ was not observed; on the quadrupole instrument, however, the $[M–Gly]^-/[M+45]^-$ ratio was 20:1, and $[M–H]^-$ had a relative abundance of 25%. We have no explanation for this exceptional behaviour.

Table 5. Relative abundances of main fragments of flavonoids with zero, one or two glycose moieties in ESI and APCI mass spectra.

Ionization mode		Glycose groups	M-H or M+H	M+FA	M-2Gly	M-Gly
APCI	NI	2	+	-	+	
		1	-	+/-		+
		0	+	-		
APCI	PI	2	+	-	-	
		1	+/-	-		+
		0	+	-		
ESI	NI	2	+	+/-	+/-	
		1	+/-	+/-		+/-
		0	+	-		
ESI	PI	2	+	-	-	
		1	+/-	-		+/-
		0	+	-		

+; 50–100%, +/-; 10-50%, -; 0–10%

MS/MS experiments

In MS/MS studies, mass-selected precursor ions are fragmented and the product ions, which provide important structural information, are recorded. In a triple-quadrupole instrument the ions that are generated in the source are continuously transmitted to the first quadrupole for precursor-ion selection, to the octapole collision cell for collision-induced dissociation (CID) and to the third quadrupole for product-ion separation and detection. The various steps take place simultaneously but are separated in space. With an ion-trap instrument, however, these steps are separated in time, with a sequence of events taking place in the same space [15].

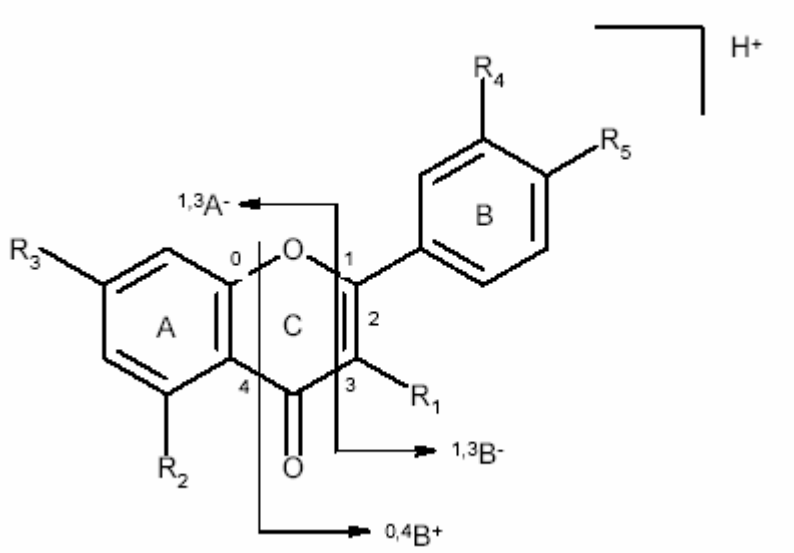


Fig. 7. Nomenclature for the different retrocyclization cleavages observed (adapted from reference 16). For explanation see text.

The MS/MS spectra of the flavonoids will be discussed for the more important NI mode, and focusing on the aglycons, because in general, apart from the loss of a glycose moiety, glycosides have the same spectra as the corresponding aglycons. One important observation, based on studying the seven aglycons, was that essentially the same results were obtained for flavones and isoflavones. Briefly, in the MS/MS spectra, $[M-H]^-$ fragments showed up in all cases and, if a methyl group is present, $[M-H-CH_3]^-$ fragments were also observed. Furthermore, fragments formed by the loss of one or more CO and/or CO_2 groups, or by retro Diels-Alder (RDA) fragmentation pathways were observed. For RDA cleavages two types of fragmentation are observed, which generate $^{x,y}A^-$ or $^{x,y}B^-$ ions. These refer to the fragments containing A- and B-rings, respectively; the superscripts x and y indicate the C-ring bonds that have been broken (see Fig. 7). For the isoflavones, $^{0,4}B^-$ RDA fragments were mainly observed, while $^{1,3}A^-$ fragments were more prominent in the case of the flavones. As an illustration, the MS/MS spectra of biochanin A are shown in Fig. 8. In Fig. 8A fragmentation on the ion-trap instrument by CID at 50% activation energy takes place in four separate steps in time (MS to MS^4). In contrast, the tandem-MS spectrum at 20-eV collision energy on the

triple-quadrupole instrument (Fig. 8B) is the result of a one-step process in which several fragment ions were formed.

The main conclusion of the study was that – for all analytes tested – the sets of MS/MS spectra recorded on the ion-trap and triple-quadrupole instruments showed that in general the same fragmentation reactions are observed. The advantage of the ion-trap is that it offers the additional possibility to ascertain precursor → product ion relationships.

Comparison with literature data

Recently, there have been many publications that deal with the determination of flavonoids in various types of samples. A brief comparison with literature values is therefore indicated. Most authors listed in Table 1 above used only one set of conditions, *i.e.* one eluent and usually only one ionization mode; moreover, in most papers, there is no information on LODs. Only six out of the twelve papers in the table used both the PI and NI modes and only three compared different ionization techniques.

From among the authors using only one ionization mode, two groups rationalized their choice, in both cases NI, by stating that although in most studies PI modes are used, NI APCI is excellent for flavonoid analysis, both regarding specific and structural information [1], while NI ESI appeared to be more sensitive and selective than PI ESI for the analysis of plant material [9]. These statements agree with our findings.

Some of the authors who used various ionization modes stated that for identification purposes spectra recorded in the PI mode were more easy to interpret because they showed more characteristic fragments than spectra recorded in NI; however, the latter type provided better detectability [2,5]. Some papers focus attention on the interpretation of the mass spectra of the flavonoids [6,7] and the various ionization modes are not discussed in much detail.

Rauha *et al.* [11] compared the use of different LC eluent compositions in APCI, IS, and also APPI, but the latter techniques is outside the scope of the present study. Using the sum of the areas of the most abundant ions in the mass spectra they found, in accordance with our results, that for PI APCI the best sensitivity is achieved with formic acid as eluent. They also noted that PI gives a much stronger background noise than NI. Seemingly in contrast with our results, these authors reported that in NI APCI the best sensitivity is obtained with AA. However, pH adjustment to 4.0 (also our optimum) was done by adding formic acid, which renders conditions closely similar to ours. In positive IS (which is comparable to our ESI mode of operation) Rauha *et al.* found that in total ion current (TIC) the best sensitivity was obtained with formic acid as an eluent while a decrease of sensitivity was observed when the formic acid concentration was lowered. The use of ammonia improved the system robustness and made it less dependent on pH; however, the presence of ammonium ions in AA decreased the sensitivity. In negative IS in TIC the best sensitivity was obtained with an eluent

containing formic acid at low pH. For AA, they found a decreased sensitivity, which might indicate ion suppression by AA or neutralization of negative charges by the ammonium anions. In line with this interpretation it was observed that increase of the concentration from 10 to 20 mM reduced the sensitivity for all flavonoids tested. The results of Rauha *et al.* cannot be straightforwardly compared with ours, since in all eluent compositions considered above ammonium ions were present to obtain an adequate LC-separation. Although the authors used other test flavonoids, the LODs were similar to ours, *viz.* 1-4 mg/l in NI APCI and 2-37 mg/l in PI APCI.

Several papers included in Table 1 provide information on detectability. LODs based on UV data of flavonoids in onion and soybean were 6-42 pmol injected [4], which corresponds with analyte concentrations of 0.08-0.63 mg/l and agrees with our findings. More importantly, one study on MS detection reports LODs in the low-µg range when using an AcN/trifluoroacetic acid gradient and full-scan PI ESI-MS detection [10]. These are fairly high values. This can probably be explained by the use of conditions, which, according to our present experiences, certainly are not optimal. Much better results were reported in another paper on the determination of flavonoids in urine using MeOH/AcN/formic acid as eluent, and NI APCI-mode detection. With selected ion monitoring, which, of course, offers a considerable gain in selectivity as well as sensitivity, LODs were 0.25–2.5 ng/ml [8]. These values are of the same order-of-magnitude as the extracted-ion-based data reported in Section 3.2 above.

4.4 Conclusions

The responses of fifteen flavonoids were compared in LC–UV and LC–MS using four different eluent compositions, while varying the pH, and four different API modes. In LC–UV, all test compounds were detected with approximately the same sensitivity; responses varied by no more than a factor of three. For LC–MS, however, much larger differences were observed; responses varied up to two orders of magnitude both between analytes and between various sets of experimental conditions. This implies that one should be cautious when interpreting data on the screening of real-life samples. In general, the eluent combination methanol/ammonium formate buffer at pH 4.0 gave the highest responses. MS responses were best in the NI modes, with APCI overall in first, and ESI in second place. It is interesting to add that the results obtained with NI APCI and NI ESI were closely similar for all aglycons. This has some practical value because, in many studies, the glycosides present in a sample are hydrolyzed prior to analysis.

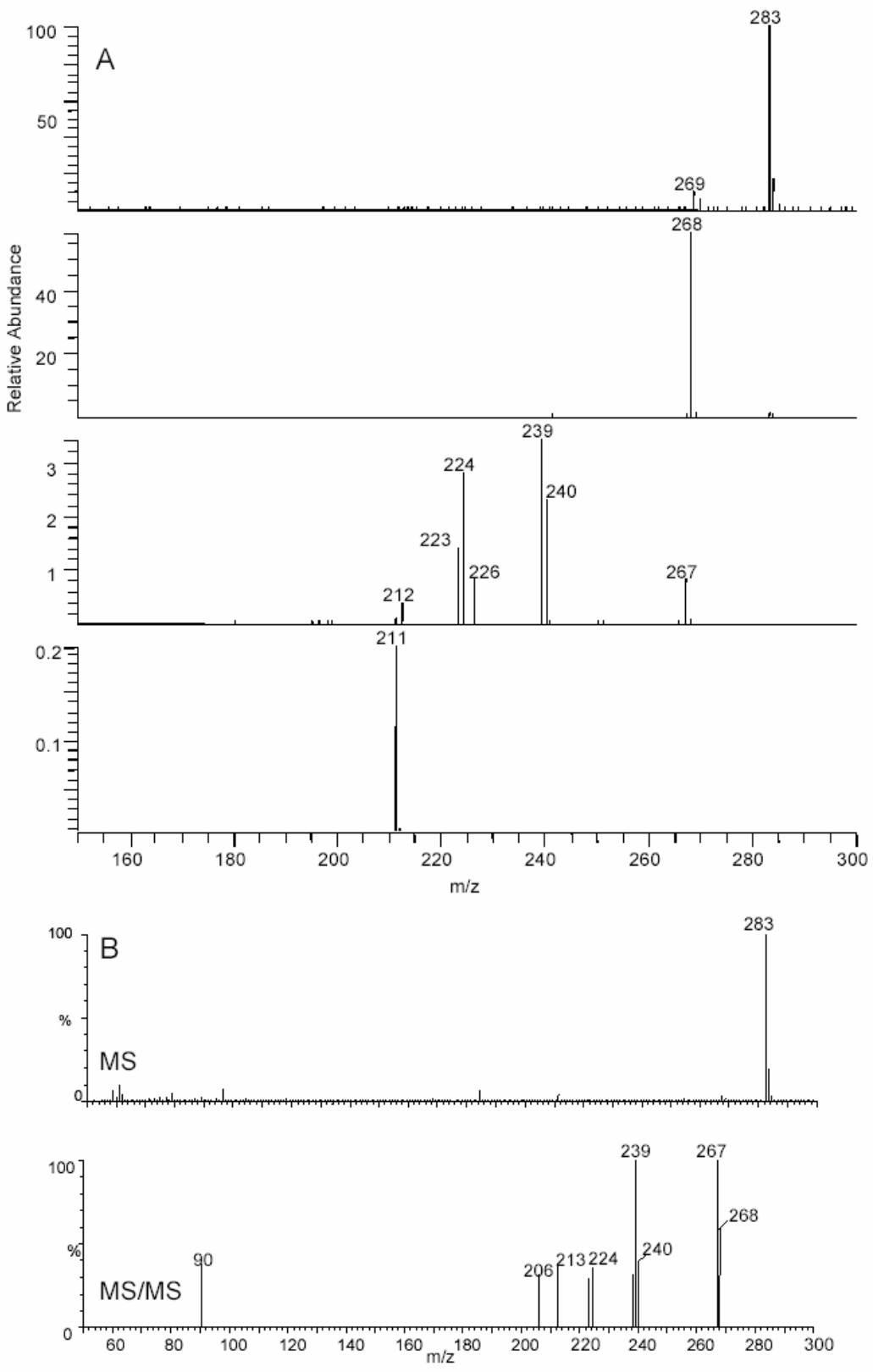


Fig. 8. MS/MS spectra of biochanin A on (A) ion-trap and (B) triple-quadrupole instruments. For LC and MS conditions, see text.

When the performance of both instruments was compared, it was found that for each of the four ionization modes, the responses were essentially the same on both machines. Three main

ions are formed; $[M–H]^-$ or $[M+H]^+$, $[M+45]^-$ and $[M–2Gly]^-$ or $[M–Gly]^-$, with formic acid adduct formation occurring in NI modes only and fragmentation being somewhat more prominent in the ESI mode. More importantly, closely similar mass spectra were generated by both MS instruments. The fragmentation reactions in MS/MS were also found to be generally the same on the ion-trap and the triple-quadrupole. Since analyte detectability on the ion-trap instrument is, today, better than it used to be some years ago [15] and is now fully comparable to that found for the quadrupole, the additional possibility to ascertain the precursor → product ion relationships seems to favour the use of the former instrument.

Finally, in the literature, not too much attention has been devoted to a systematic comparison of different ionization modes and/or LC eluents for flavonoids. Still, a close scrutiny of the published data reveals that the majority of these agrees with our preferred approach, the use of MeOH/AF (pH 4.0) with NI APCI mass spectrometry. Currently, the method is being used – on the ion-trap instrument – to detect and identify flavonoids, both target compounds and unknowns, in wetland plants.

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5. Flavonoids in Leguminosae: Analysis of extracts of *T. pratense* L., *T. dubium* L., *T. repens* L., and *L. corniculatus* L. leaves using liquid chromatography with UV, mass spectrometric and fluorescence detection[1]

Summary

Reversed-phase LC on C18 bonded silica with a methanol–ammonium formate gradient was used to determine the main flavonoids in leaves of four species of the Leguminosae family. The detection modes were diode-array UV absorbance, fluorescence, and (tandem) mass spectrometry. LC–UV was used for a general screening, sub-classification, and the calculation of total flavonoid contents. LC–FLU was included to identify isoflavones on the basis of their native fluorescence. Most structural information regarding aglycons, sugar moieties, and acidic groups was derived from LC–MS in both the full-scan and extracted-ion mode, using negative-ion atmospheric pressure chemical ionization. MS/MS did not provide much additional information, because the same fragments were observed as in full-scan MS.

In *T. pratense* and *T. repens*, the main constituents were flavonoid glucoside–(di)malonates, while *T. dubium* and *L. corniculatus* mainly contained flavonoid (di)glycosides. Satellite sets comprising an aglycon, the glucoside and glucoside–malonates or –acetates, were abundantly present only in *T. pratense*. Generally speaking, the main aglycons and sugars in the four plant species are surprisingly different. In addition, while the results for *T. pratense* are similar to those reported in the literature, there is little agreement in the case of the other species. Finally, total flavonoid contents ranged from 50–65 mg/g for *L. corniculatus* and *T. dubium*, to 15 mg/g for *T. pratense* and only 1 mg/g for *T. repens*.

[1]E. de Rijke, H. Zappey, F. Ariese, C. Gooijer, U.A.Th. Brinkman, *Anal. Bioanal. Chem.* 378 (2004) 995.

5.1 Introduction

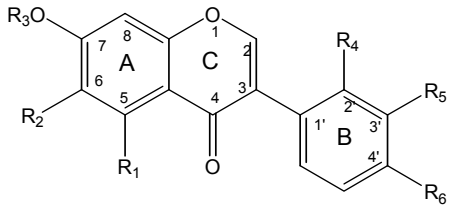
Flavonoids are the largest group of secondary metabolites in plants [1]. They are usually present as glycosides, i.e., provided with one or more sugar substituents such as galactose, rhamnose or glucose, or glucoside–malonate. Sugar substitution on the flavonoid skeleton occurs through hydroxyl groups in the case of O-glycosides or directly to carbon atoms in ring A in C-glycosides. Structures and abbreviations used in this paper are given in Scheme 1. Flavonoid glycosides and free aglycons play an important role in plants as defense and signaling compounds in reproduction, pathogenesis, and symbiosis [2,3]. They also act as UV protectants in plant cells [4], as pigment sources for flower coloring compounds [5] and play an important role in interactions with insects [1]. They also affect human and animal health because of their role in the diet, which is ascribed to their antioxidant properties [6], estrogenic action [7], and a wide spectrum of antimicrobial and pharmacological activities [8,9].

Plants from the Leguminosae family are known for their rich flavonoid content. In this paper, we investigate four plants from this family, *T. pratense* L. (red clover), *T. dubium* L. (small hop clover), *T. repens* L. (white clover), and *L. corniculatus* L. (common bird's-foot trefoil). They all commonly occur in wetlands and are easy to obtain. To determine the main flavonoid constituents in the leaves of the four species, we will apply previously optimized reversed phase liquid chromatography coupled to (tandem) mass spectrometry (LC–MS(/MS)) [10] and UV absorbance combined with fluorescence detection (LC–UV–FLU) [11] methods.

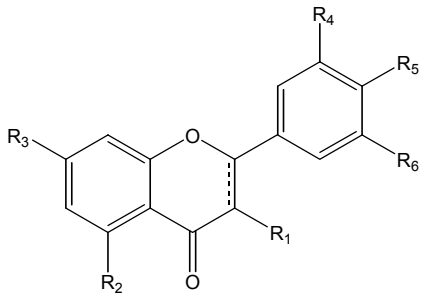
To date, much attention has been paid only to *T. pratense*, which is a well-known source of flavonoids. Many flavonoids and their glucoside and glucoside–malonate derivatives have been identified in *T. pratense* by using LC–UV or LC–MS [12–17]. *L. corniculatus* and *T. repens* are important forage legumes that improve the nutrition of sheep by reducing ruminal degradation of plant protein and increasing crude protein flow to the intestine [18]. In several papers the presence of flavonoids, such as various quercetin and kaempferol (di)glycosides, in *T. repens* has been reported [11,19–23]. Most of the studies dealing with *L. corniculatus* and a single paper devoted to *T. dubium* were published a rather long time ago and should therefore be used with some caution. Relevant details will be presented in the pertinent sections below.

In the literature on flavonoid analysis in plants or food, most authors use only one analytical method and, in the case of LC–MS, usually only one or two ionization modes. In this paper, we compared four ionization modes—atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI), positive ionization (PI) and negative ionization (NI) modes—using a *T. pratense* extract and analyzed extracts from *T. pratense*, *T. dubium,*

T. repens, and *L. corniculatus* leaves by means of LC–DAD UV combined with fluorescence (FLU) detection and LC–NI APCI-MS(/MS). Although the literature also describes analysis of extracts of other parts of these plants, in this study we only investigated extracts of leaves because they contain the highest amount of flavonoids. The main aim of the study was to record the flavonoid profiles of the four closely related species and to compare them while using the same set of analytical techniques.



Isoflavone	Abbrev.	R_1	R_2	R_3	R_4	R_5	R_6	MW
Afrormosin	A	H	OCH_3	H	H	H	OCH_3	298
Biochanin A	B	OH	H	H	H	H	OCH_3	284
Calycosin	C	H	H	H	H	OH	OCH_3	284
Daidzein	D	H	H	H	H	H	OH	254
Formononetin	F	H	H	H	H	H	OCH_3	268
Genistein	G	OH	H	H	H	H	OH	270
Glycetein	Gl	H	OCH_3	H	H	OH	OH	284
Irilin B	IB	OH	OCH_3	H	OH	H	H	300
Irilone	I	OH	O–	CH_2–	H	H	OH	298
3-Methylorobol	3M	OH	H	H	H	OCH_3	OH	300
Pratensein	P	OH	H	OH	H	OH	OCH_3	300
Pseudobaptigenin	Ps	H	H	H	H	O–	OCH_2–	282
Texasin	TG	H	OH	H	H	H	OCH_3	284



Flavone	Abbrev.	R_1	R_2	R_3	R_4	R_5	R_6	C_2-C_3	MW
Hesperidin	HG2	H	OH	7-O-β-D-Neohesp	OH	OCH_3	H	single	610
Hesperetin	H	H	OH	OH	OH	OCH_3	H	single	302
Naringin	NG2	H	OH	7-O-β-D-Neohesp	H	OH	H	single	580
Naringenin-7-glucoside	NG	H	OH	7-O-β-D-Glu	H	OH	H	single	434
Naringenin	N	H	OH	OH	H	OH	H	single	272
Rutin trihydrate	RG2	3-O-β-D-Neohesp	OH	OH	OH	OH	H	double	610
Kaempferol	K	OH	OH	OH	H	OH	H	double	286
Quercetin	Q	OH	OH	OH	OH	OH	H	double	302
Myricetin	M	OH	OH	OH	OH	OH	OH	double	318
Isorhamnetin	Is	OH	OH	OH	OCH_3	OH	H	double	316

Scheme 1. Structures of the flavonoids studied and their acronyms.

5.2 Experimental

Materials

Biochanin A, sissotrin, and daidzin were purchased from Indofine (Somerville, NJ, USA), and daidzein, genistin, genistein, formononetin, and ononin from Roth (Karlsruhe, Germany). Methanol and formic acid were from J.T. Baker (Deventer, the Netherlands), ammonium formate and white quartz sand from Aldrich (Steinheim, Germany). Tris(hydroxymethyl)aminomethane (TRIS) was purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, the Netherlands). The extracts were prepared from *T. pratense*, *T. dubium, T. repens,* and *L. corniculatus* leaves from plants collected in a wetland in the Netherlands (Horstermeerpolder). The plants were grown in a greenhouse. All extracts were filtered over a 0.45 μm filter before injection.

Extraction

Extraction of the leaves was modified according to a previous method [11]. Fresh leaves (500 mg) were well ground with 3 mL of aqueous 350 mmol L^{-1} TRIS solution. A few grams of white quartz sand and 10 mL of methanol–water (9:1, *v/v*) were added, and the extract was stirred for 1 min. The extract was filtered over a Büchner funnel and 10 mL of methanol–water (9:1, *v/v*) was added to the residue. This procedure was repeated and the combined extracts were filtered over a 0.45 m filter and stored at –20 °C until use. Under these conditions, the extracts are stable for at least 2 weeks. However, the extraction must be performed as quickly as possible because, at room temperature, malonyl–glucoside flavonoids in freshly picked leaves undergo hydrolysis to their glucosides [11]. TRIS solution, which functions as a β-glucosidase inhibitor, is added to prevent this. Of the extracts, 5 mL were evaporated under a gentle stream of nitrogen and the residue was dissolved in 1 mL of methanol–water (3:7, *v/v*).

Instrumentation

LC–DAD UV–FLU. LC was performed on a HP 1050 LC system (Hewlett Packard, Waldbronn, Germany) with a photo diode array detector (set at 290, 265, and 250 nm) and an Applied Biosystems (Foster City, CA, USA) Model 980 programmable fluorescence detector. Excitation was at 250 nm, while an emission cut-off filter blocking wavelengths shorter than 450 nm was inserted. For separation a 250×4.6 mm i.d. 5 μm Zorbax SB-C18 LC column was used, and for elution a gradient of methanol and 10 mM ammonium formate buffer, pH 4.0, with a flow rate of 1.0 mL/min. The gradients for *T. pratense* L., *T. dubium* L. and *T. repens*

L. extracts are presented in Table 1A. For *L. corniculatus* a somewhat less steep gradient was needed (Table 1B) to separate the main components in the extract. Before use all solvents were filtered over a 0.45 μm filter and degassed with helium. Sample injection was performed with a Midas autosampler (Spark Instruments, Emmen, the Netherlands). The injection volume was 10 μL. For details of the procedure, see [11].

LC–APCI/ESI-MS. For LC–MS a Shimadzu (Princeton, NJ, USA) LC system was used consisting of two LC-10A pumps, a SCL 10A controller unit, a DGU-14A degasser, a SIL-10AD auto-injector and a SPD-10A UV detector (set at 265 and 290 nm). The set-up was coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The injection volume, column and gradients were the same as in LC–UV–FLU. APCI and ESI spectra were obtained in both the NI and the PI mode in the *m/z* 150–650 range. The capillary temperature for ESI was set at 275 °C; with APCI the capillary and vaporizer temperatures were 250 °C and 450 °C, respectively. After optimization for all flavonoids, the capillary voltage was set at 4 V for PI ESI, –4 V for NI ESI, 46 V for PI APCI and –12 V for NI APCI. The APCI corona discharge current was 10 μA. The sheath-gas (nitrogen) flow rates for APCI and ESI were 80 and 40 units, respectively, which corresponds to approx. 200 and 20 L/h, and the auxillary gas (nitrogen) flow rate was 20 units for both APCI and ESI. For MS/MS, the activation energy was set at 50%. For APCI, the LC flow was directed into the mass spectrometer without stream splitting, while for ESI the LC flow was split and 0.2 mL/min was sent to the mass spectrometer. For details of the procedure, see [10].

Table 1 Reversed-phase LC gradients (in vol. %) used for flavonoid analysis in (A), *T. pratense*, *T. dubium* and *T. repens*, and (B) *L. corniculatus*

Time (min)	% methanol	% buffer
A		
0	30	70
5-10	45	55
15	50	50
25	55	45
30	60	40
35-40	80	20
42	30	70
B		
0	30	70
5	40	60
10	45	55
15	50	50
35-40	80	20
40-47	30	70

5.3 Results and discussion

Analytical performance

Four ionization modes (PI and NI APCI; PI and NI ESI) were tested using a *T. pratense* extract and optimized LC conditions. In the PI modes, background signals were higher than in the NI modes. Overall, the analyte responses were best in the NI modes, with APCI in first, and ESI in second place. The only exception was formononetin, for which the order is reversed. In this study, LC–NI APCI-MS was used throughout.

Previously, we determined the analytical performance of the LC–MS method [10] for 15 flavonoids (NG2, HG2, FG, BG, H, K, F, B, DG, GG, NG, RG2, D, N, and G, respectively; for full names see Scheme 1) and full-scan detection limits (LODs, S/N=3) were found to be 0.1–20 mg/L (R^2 values of calibration plots between 0.990 and 0.999 in all cases). The somewhat high LODs (median value: 3 mg/L) were caused by the low response of some of the flavonoids in the mixture and by the use of full-scan acquisition. In this work we determined LODs in the time-scheduled selected ion monitoring (TS SIM) mode for a standard mixture of seven flavonoids that are common in *T. pratense* (B, BG, DG, D, GG, FG, and F); the $[M–H]^-$ of the aglycons were used for quantification. In SIM, LODs were found to be 1–45 μg/L (median value 8 μg/L). The R^2 values (calibration range 0.1–20 mg/L, 5 data points in triplicate) improved to between 0.993 and 0.999 in all cases. For LC–UV (250 nm), LODs were 0.08–0.6 mg/L (median value: 0.25 mg/L), with R^2 values (in the same calibration range) between 0.995 and 0.999. We can conclude that with MS detection operating in the TS SIM mode the lowest LODs are obtained, but there is also a greater variation between the individual flavonoids. Because the linearity of UV is better than that of MS, especially in the lower concentration ranges, peak areas in UV were used for quantification. In fact, UV detection enabled us to get a complete overview of the main flavonoids in all extracts studied below, and to estimate the total flavonoid contents in a rather simple way. This is based on the fact that the wavelengths of maximum absorption for flavonoids with the same chromophoric group are essentially the same, as is obvious from the data in Table 2 (which will be considered in detail below).

Table 2 Maxima of the UV spectra of the analyte peaks in the extracts.

Plant	Compound	Max 1 (nm)	Max 2 (nm)	Flavonoid class*
T. pratense	1, 2, 3, 7	250	300	Isoflavone
	4, 5, 6, 8	260	335	Isoflavone
T. repens	9	233	335	Isoflavone
	10	230	340	Isoflavone
	11	230	335	Isoflavone
L. corniculatus	13, 14	265	350	Flavone
T. dubium	15, 16, 17	255	355	Flavone

*Based on literature data [25].

***T. pratense* L.**

Fig. 1 shows LC–UV and LC–FLU chromatograms of a *T. pratense* extract. Eight prominent peaks show up at retention times between 13 and 35 min, with peaks 1–3 and 7 displaying native fluorescence. As we discussed in a previous paper [24], only isoflavones that do not contain a hydroxy group in the 5-position (see Scheme 1) show fluorescence. The spectral properties of the various peaks are listed in Table 2. Compounds 1–3 and 7 have essentially identical UV spectra and the same is true for the UV spectra of compounds 4–6 and 8. Each flavonoid sub-class (e.g., isoflavones, flavones, flavanones) has a characteristic UV spectrum [25]. For example, isoflavones have their first maximum around 255 nm and their second maximum between 300 and 340 nm, while flavones have their first maximum around 260 nm and the second one—which is more pronounced than for the isoflavones—around 360 nm. From this we can conclude that all eight compounds are isoflavones, with compounds 1–3 and 7, and compounds 4–6 and 8 each having the same basic structures.

Fig. 2 shows the full-scan and two extracted-ion LC–MS chromatograms of the same extract as used in Fig. 1. The extracted-ion traces allow us to distinguish compounds with the same aglycon mass. The peaks in the center trace of Fig. 2 have a formononetin-based structure (*m/z* 267) and differ from each other in having different glucoside and/or malonate substituents. In the same way the main peaks in the lower trace of Fig. 2 have biochanin A (*m/z* 283) as their aglycon. Each extracted-ion trace contains aglycon, glucoside, glucoside–malonate, and glucoside–malonate isomer peaks, as will be explained below. In the present study, ten of such 'satellite sets' were found in *T. pratense*, which will be discussed in the next section. Here we focus our attention on the main peaks, which are the compounds containing formononetin (F) and biochanin A (B).

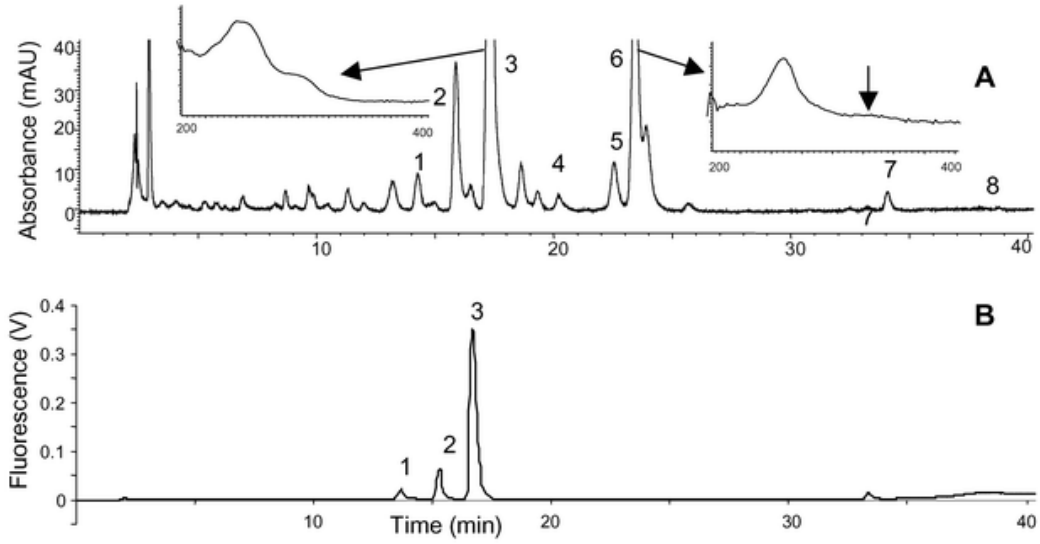


Fig. 1 Reversed-phase LC–UV_{250} (*A*) and reversed-phase LC–FLU (ex, 250 nm, em >450 nm) (*B*) of *T. pratense* extract. *Inserts*: UV spectra of peaks 3 and 6.

The inserts of Fig. 2 show the mass spectra of the eight flavonoid peaks. The mass spectra of peaks 1–3 and 7 all contain *m/z* 267 and 252; the mass difference is indicative for the loss of a CH_3 group. The spectra of peaks 1–3 also contain *m/z* 475 which points to a glucose moiety plus a formic acid (FA) adduct (162+46); a minor peak at *m/z* 431 for the glucoside is also observed in the spectra of peak 1 and 2. The presence of FA adducts under the experimental conditions used was discussed earlier [10]. The spectra of peaks 1 and 3 also

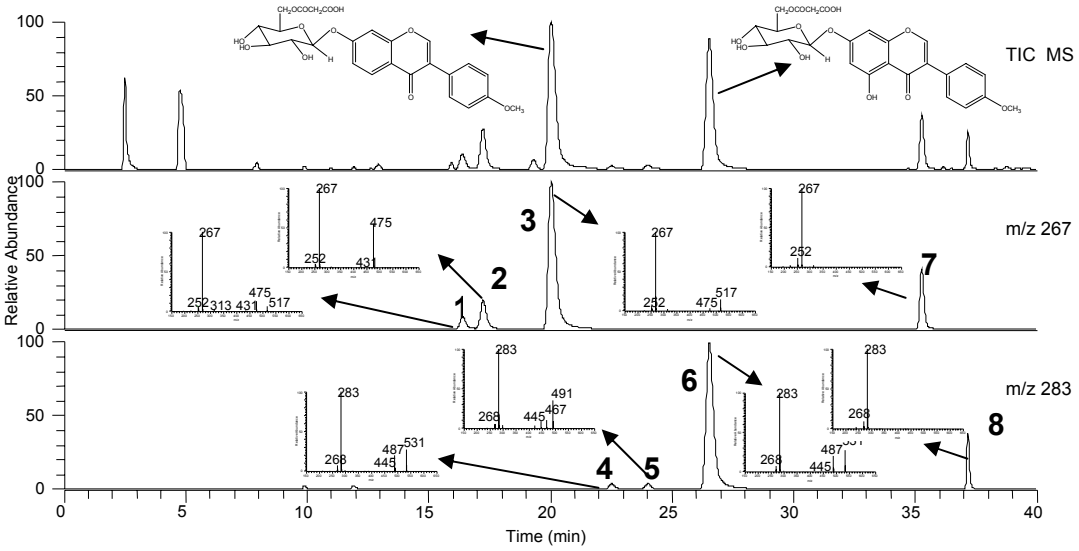


Fig. 2 Full-scan and extracted-ion LC–NI APCI-MS of *T. pratense* extract, with mass spectra of peaks 1–8 and structures of main glucoside–malonate peaks 3 and 6. For details, see text.

contain *m/z* 517, which is consistent with a malonate group. On the basis of the combined data, the peaks can be attributed to the isoflavones, (3) formononetin–7-*O*-β-D-glucoside–6″-*O*-malonate (FGM), (1) an isomer of FGM, (2) formononetin–7-*O*-β-D-glucoside (FG) and (7) F.

A similar analysis of the mass spectra of peaks 4–6 and 8 reveals that, in this instance, next to the loss of a CH_3 group, the presence of a glucose moiety with and without an FA adduct (peak 5) and a malonate group (peaks 4 and 6) is indicated. The peaks can be attributed to the isoflavones, (6) biochanin A–7-*O*-β-D-glucoside–6″-*O*-malonate (BGM), (4) an isomer of BGM, (5) biochanin A–7-*O*-β-D-glucoside (BG), and (8) B. The elution order of FGM and BGM and their respective isomers is based on earlier reports [12,13], with the isomers having the smaller peak heights and the shorter retention times.

For further confirmation of the proposed structures, MS/MS was performed on the $[M–H]^-$ of each peak, at activation energies varying between 20 and 50%. However, the MS/MS spectra did not show any fragment ions other than those observed in full-scan MS. MS/MS was also performed on the main fragment ions in the full-scan spectra—*m/z* 267 for peaks 1–3 and 7, and *m/z* 283 for peaks 4–6 and 8—for all eight compounds. Fig. 3 shows the

MS/MS spectra of peaks 3 and 6. In both spectra $[M–15]^-$ fragments, corresponding to the loss of CH_3, $[M–44]^-$ fragments, corresponding to the loss of CO_2, and $[M–59]^-$, corresponding to the loss of both CH_3 and CO_2, are observed. The MS/MS spectra of peaks 1, 2 and 7 (*m/z* 267) were found to be very similar to that of peak 3, which confirms that all peaks have the same aglycon mass; the same was true for peaks 4–6 and 8 (*m/z* 283). Also, after increasing or decreasing the activation energy the spectra did not show any differences, confirming that the basic structure (*m/z* 267 or 283) is the same. In other words, LC–MS/MS confirms that the isomer peaks, 1 and 4, have F- and B-based structures, respectively, but no additional information is provided. Most likely, they differ from FGM and BGM as regards the position of the glucose and/or malonate groups. As yet, we do not know which positions are involved; no relevant information is provided in the literature.

The UV spectra of FGM, FG, and F are closely similar, and the same is true for BGM, BG, and B. Therefore, for quantification of compounds 1–3 and 7, the UV_{265} calibration plots of FG and F were used, and for compounds 4–6 and 8, the UV_{265} calibration plots of BG and B. Corrections were made for the difference in molecular weight between glucoside–malonate, glucoside, and aglycon. The concentrations of FGM and BGM were calculated to be about 5 mg/g of leaves, while the concentrations of the other six analytes were 0.1–1 mg/g of leaves. The total flavonoid content was about 15 mg/g of leaves. In the literature, only Krenn et al. [17] have quantified isoflavones in red clover. They determined the concentrations of four aglycons (F, B, G, and D) after hydrolytic extraction; their concentrations were 0.1 mg/g (D and G), 2 mg/g (B), and 3 mg/g (F); the total content was 5 mg/g. The authors studied different cultivars (which were not further specified) and found that their B and F content varied between 0.03 and 0.3%. The lower concentrations in the quoted study compared with the present one may be due to the extraction conditions: the elevated temperatures used for hydrolysis [17] can cause partial degradation of the flavonoids in the extracts.

Satellites

As was briefly mentioned above, many of the flavonoids in *T. pratense* occur as satellite sets of aglycons, glucosides, glucoside–malonates and, in some cases, glucoside–acetates. When screening an LC–MS chromatogram these satellite sets are easily recognized by their (identical) aglycon mass. This can be seen in Fig. 2 for the satellite sets of F and B which consisted of the glucoside–malonate, an isomer, the glucoside and the aglycon. A total of 13 satellites sets was found in *T. pratense*, comprising 40 isoflavones. They are listed in Table 3. Satellite sets were not found for all flavonoids. For example, for the flavones apigenin and kaempferol only the glucosides were found, and for 3-methylquercetin only the aglycon. To the best of our knowledge, this is the first discussion of satellite sets.

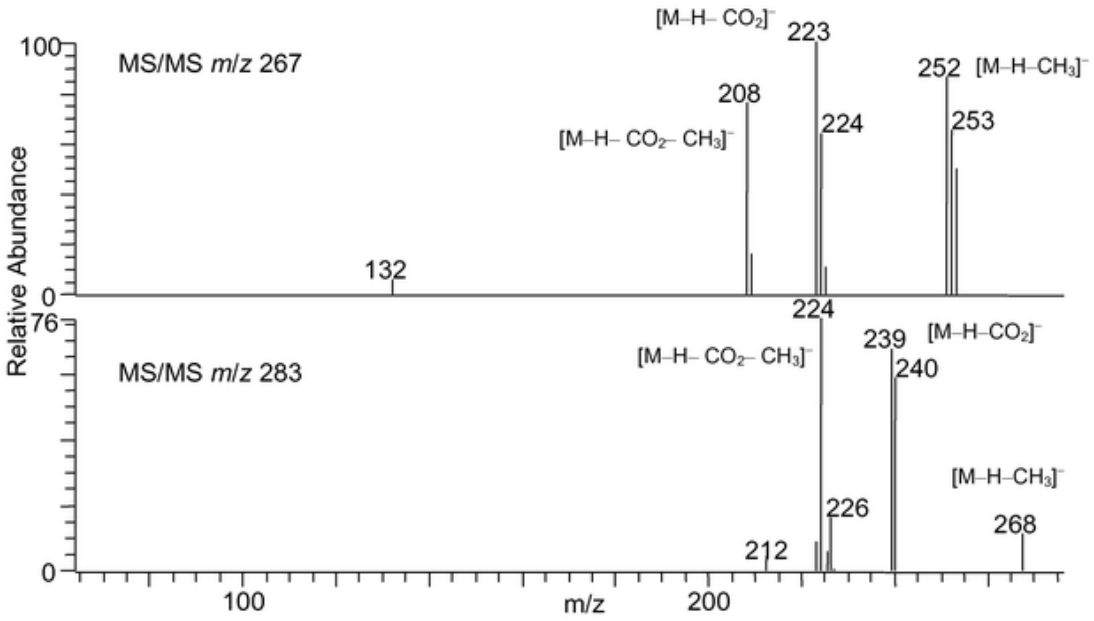


Fig. 3 LC–NI APCI-MS/MS of peaks 3 (*m/z* 267) and 6 (*m/z* 283) of the *T. pratense* extract (see Fig. 2). Activation energy: 50%.

In the literature, two papers are dedicated to flavonoid glucoside–malonates in *T. pratense*. It is interesting to quantitatively compare their results with our findings. Lin et al. [12] identified 23 flavonoids in extracts of leaves, with seven different aglycons and reported the presence six glucoside–malonates. Klejdus et al. [13] identified 49 isoflavones, also in leaves, based on 20 aglycons. Six glucoside–acetates and 14 glucoside–malonates were detected. From among these glucoside–malonates, nine were identified also by us. From the three glucoside–acetates identified by us, Klejdus et al. only found irilone–glucoside–acetate. On the other hand, the other five glucoside–acetates reported by these authors (DGA, FGA, PsGA, PGA, and BGA) were absent from our extracts, as was demonstrated by the pertinent extracted-ion traces in LC–MS. One difference is that extensive dual-SPE sample treatment was used in ref. [13], while there was no pretreatment at all in our study. Another explanation may be differences in natural environment, growth conditions, etc., which are not given in any of the studies quoted so far. In our analyses, the glucoside–malonate peaks are the highest ones in most cases, as was also observed in ref. [12]. However, in ref. [13] the glucosides generally are most prominent. Most likely this is caused by the extraction conditions: Lin et al. dried the leaves and performed the extraction at room temperature, while Klejdus et al. used a somewhat higher temperature (30 °C), which will promote hydrolysis of the glucoside–malonates. In our extracts, the glucoside–malonate peaks are relatively high due to the protective action of TRIS. More importantly, despite the somewhat different concentrations, the eight main constituents found in this study are the same as those reported in the literature [12,13].

Table 3 Satellite sets in *T. pratense.*

Isoflavone	Mol.wt. of A	A	G	GM	GA
Afrormosin	298	+	+		+
Biochanin A	284	+	+	+*	
Calycosin	284	+	+	+	
Daidzein	254	+	+	+	
Formononetin	268	+	+	+*	
Genistein	270	+	+	+*	+
Glycetein	284	+	+	+	
Irilin B	300	+	+		
Irilone	298	+	+	+	+
3-Methylorobol	300	+		+	
Pratensein	300		+	+	
Pseudobaptigenin	282	+	+	+	
Texasin	284	+	+		

*Isomer found, *A* aglucon, *G* glucoside, *GM* glucoside–malonate, *GA* glucoside–acetate

***T. repens* L.**

The results of the LC–UV and LC–FLU analysis of a *T. repens* extract are shown in Fig. 4. In the LC–UV_{250} trace several peaks show up of which three show native fluorescence at retention times between 12 and 18 min. These three fluorescent compounds are most likely isoflavones—because they show fluorescence at an emission wavelength above 450 nm, and have no OH group at the 5-position, as is evident from their UV characteristics (cf. Table 2). This means that they exhibit a large Stokes' shift, which is characteristic for fluorescent isoflavones [24].

On the basis of their UV and mass spectra, the prominent cluster of peaks between 2 and 6 min cannot be attributed to flavonoids, cf. [25]. The same is true for the other peaks at retention times below 10 min, which are evident in the UV trace.

LC–NI APCI-MS of a *T. repens* extract shows four main compounds (peaks 9–12) in the full-scan trace (Fig. 5A). Three of these are part of a satellite set and all contain *m/z* 353 (Fig. 5B). In the mass spectrum of peak 9, fragment ions are observed at *m/z* 561, 353, 267 and 252, the latter two ions being rather weak. This indicates loss of a CH_3 group (*m/z* 267/252), the presence of a malonate group (*m/z* 353/267) and that of a glucose moiety plus an FA adduct (*m/z* 561/353). Consequently, peak 9 can most likely be attributed to a F glucoside–malonate, with the strong fluorescence supporting the proposed aglycon structure. A (small) peak due to $[M–H]^-$ at *m/z* 515 (561–FA) can indeed be observed in the mass spectrum. This is consistent with what is observed for other glucosides such as FG (Fig. 2, peak 2) with which the adduct peak is also much more prominent than the $[M–H]^-$ peak. Although peaks 1 and 3 in Fig. 1 are also attributed to F glucose–malonates, the mass spectrum of peak 9 differs from their mass spectra by the addition of a FA group on the glucose–malonate fragment and a malonate group on the aglycon fragment. The mass spectra

of peaks 10 and 11 in Fig. 5 also display an *m/z* 601 peak. This suggests *m/z* 515 plus a malonate group, i.e., they indicate the presence of two glucoside–dimalonates. Because a *m/z* 353 fragment is observed for peaks 10 and 11, this means that the malonate group is either attached to the aglycon skeleton (M–F–G–M), or—what is more likely—that the second malonate group is attached to the first malonate, with rearrangement taking place during fragmentation, which causes the second malonate group to associate with the aglycon (F–G–M–M→F–M+G–M). In the literature, this rearrangement has been observed for diglycosides [26]. From the literature [23], only dimalonates of anthocyanins (with a structure similar to flavones but without the C-ring carbonyl group) are known. To the best of our knowledge isoflavone glucoside–(di)malonates were not detected before in *T. repens*.

As with *T. pratense*, MS/MS analysis did not provide much useful additional information. The closely similar spectra of peaks 9–11 obtained with *m/z* 353 as parent ion merely confirmed the identity of the base structures of the three compounds. Their concentrations are each about 0.3 mg/g of leaves, as calculated from the UV_{250} calibration plots of FG and F, or some 16-fold lower than for the main flavonoids in *T. pratense*.

Finally, the MS spectrum of peak 12 only features *m/z* 422 and its FA adduct at *m/z* 467. The peak does not show up in the UV and fluorescence traces (cf. Fig. 4) and in the MS/MS spectrum of *m/z* 422 no further fragmentation is observed. Because all flavonoids clearly show a UV signal and no fragmentation is observed under the operating conditions used, the compound is not a flavonoid, but its identity is as yet unknown.

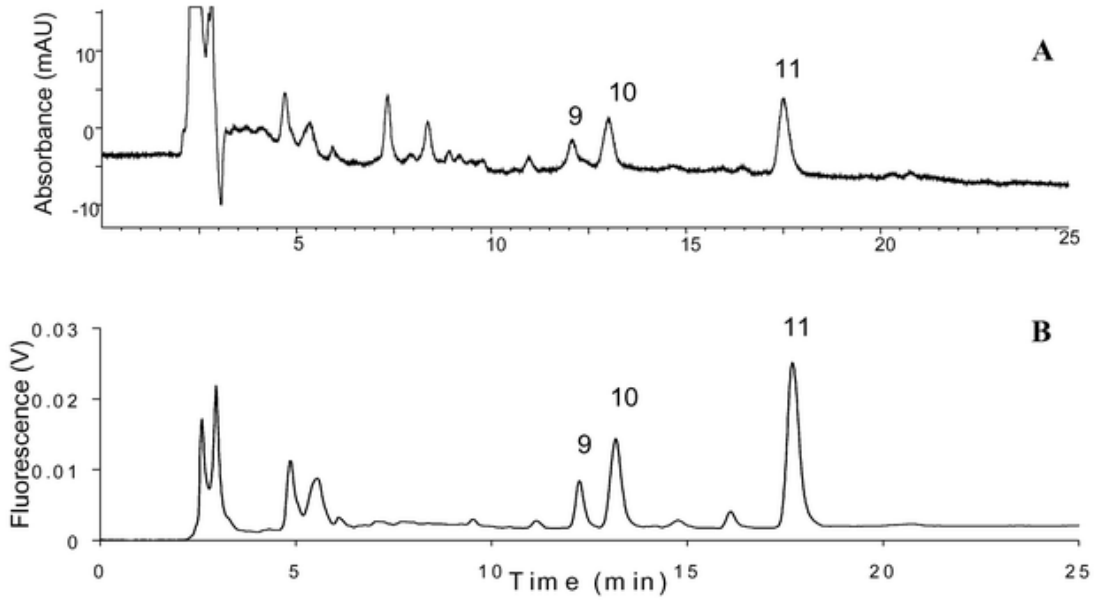


Fig. 4 LC–UV_{250} (*A*) and LC–FLU (ex, 250 nm, em >450 nm) (*B*) of *T. repens* extract.

The presence of flavonoids in *T. repens* has been reported in only a few papers [20–22,27]. Schittko et al. [27] isolated three main flavonoids from *T. repens* flowers; the 3-*O*-

galactosides of myricetin (M) and quercetin (Q) and Q–(6″-*O*-acetyl)–3-*O*-galactoside. They used LC–UV, with subsequent off-line PI ESI-MS and NMR for identification. Minor constituents were Q, two kaempferol (K) derivatives, and two unknown isoflavones which were not further specified. Hofmann et al. [20], who used LC–UV and off-line NMR, isolated Q–(3-*O*-β-D-xylopyranosyl)–(1→2)-β -D-galacatopyranoside and K–(3-*O*-β-D-xylopyranosyl)–(1→2)-β -D-galacatopyranoside as the main flavonoids from UV-B-irradiated *T. repens* leaves. Mathesius [21] identified 7,4′-dihydroxyflavone, a 7,4′-dihydroxyflavone derivative and formononetin in a *T. repens* root extract using LC–UV. Only Vetter [22] quantified flavonoid aglycons in extracts of hydrolyzed *T. repens* leaves using LC–UV. The concentrations of D, G, F, and B in leaves, stems, and flowers varied between 0.001 and 0.085 mg/g—the total concentration in the leaves being 0.03 mg/g—which is one order lower than in our extracts. Again, a partial degradation due to the elevated temperatures used for hydrolysis may be one explanation for these differences.

Careful screening of the LC–MS data by means of extracted-ion traces did not reveal even traces of G, B, M, K, or Q in our *T. repens* extract. Actually, as the above brief overview shows, the flavonoids found in *T. repens* vary greatly in the literature, the only common results being the identification of Q glycosides in both flowers [27] and leaves [20], and the detection of F by Vetter [22] and in the present study. As also referred to earlier, the published papers do not provide any information regarding environmental factors or growth conditions that may explain the observed differences.

***Lotus corniculatus* L.**

The LC–UV chromatogram of *L. corniculatus* (Fig. 6) shows two main compounds in the 13–14 min retention time region with flavonoid-type UV spectra (peaks 13 and 14). Based on their spectral properties listed in Table 2 the peaks can be attributed to flavones, which have their first maximum around 260 nm and their second one around 360 nm [25]. LC–FLU was also recorded, but no relevant peaks were observed, except for a cluster of peaks around 5 min which, according to their UV and mass spectra, cannot be attributed to flavonoids.

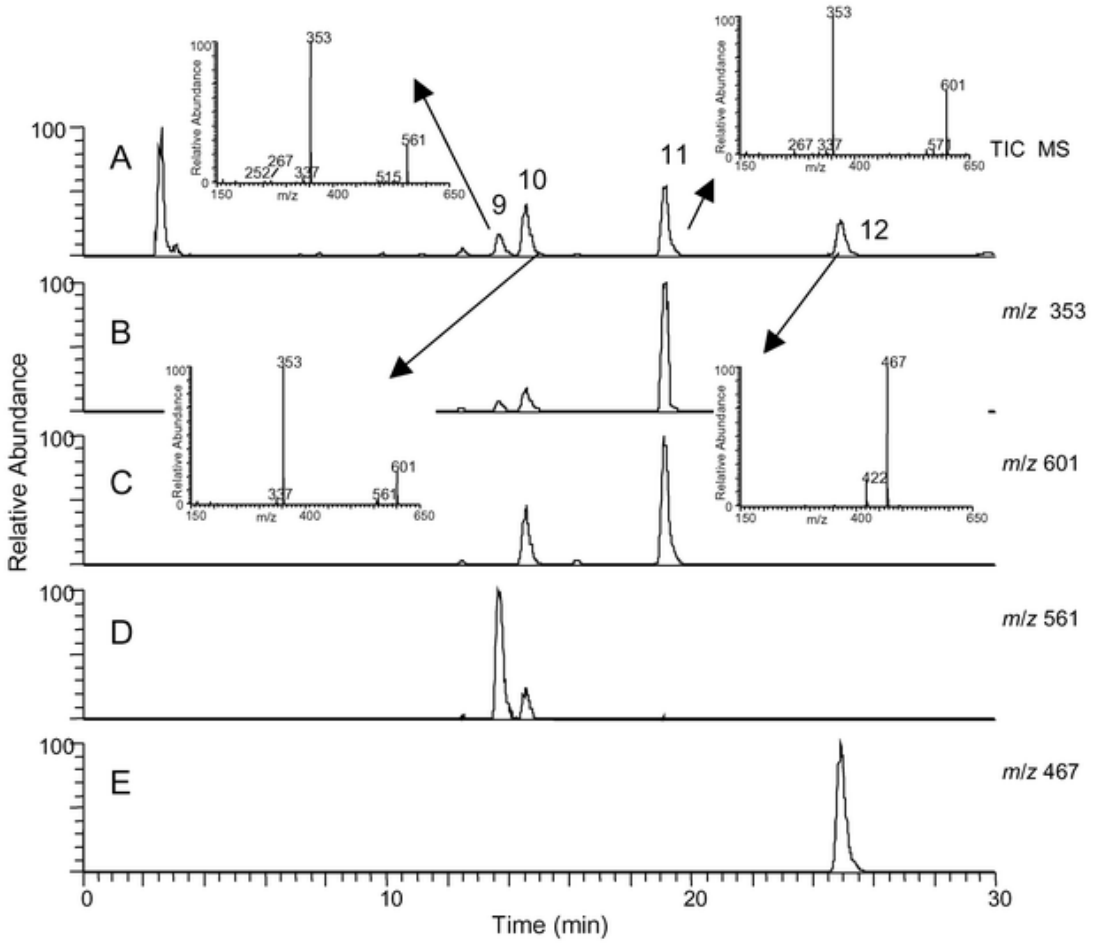


Fig. 5 Full-scan (A) and extracted-ion (B–E) LC–NI APCI-MS of *T. repens* extract; inserts: mass spectra of peaks 9–12.

The corresponding LC–MS chromatogram is shown in Fig. 7. In the full-scan trace, the same two main peaks show up, which display *m/z* 593, 447, 431, and 285 (extracted-ion traces B–E) as prominent fragment ions. The mass spectra of peaks 13 and 14 are included in Fig. 7. Some very distinct fragmentations are observed (for explanation of terms, see Fig. 8). The Y_1^- ion in the mass spectra of Fig. 7 is formed after loss of a rhamnose unit, and the Y_0^- ion after loss of another glucose unit. The Y^* ion in this figure corresponds to the loss of the

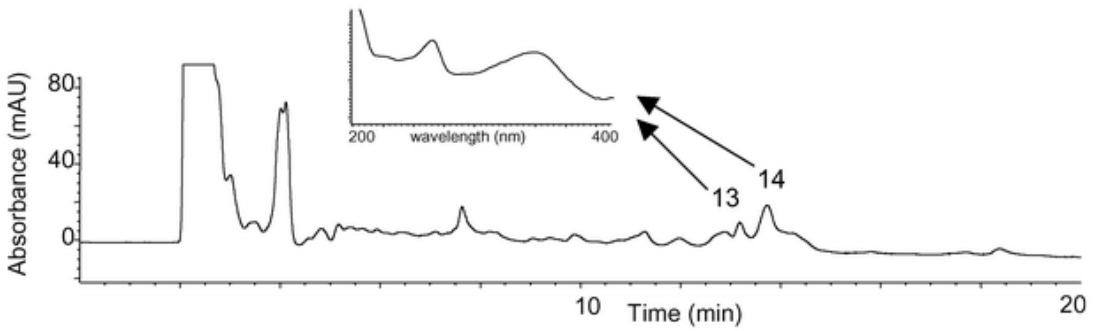


Fig. 6 LC–UV_{250} trace of *L. corniculatus* extract, with (identical) UV spectra of peaks 13 and 14.

glucose residue (162 amu) from the $[M–H]^-$ ion, as described by Ma et al. [26], and is only observed for flavonoid *O*-rutinosides (*O*-rhamnosyl-(α 1→6)-glucose) and *O*-neohesperidosides (*O*-rhamnosyl-(α 1→2)-glucose). This fragment is present only in the case of 3-*O*-flavonols and not 7-*O*-flavonols. That is, peaks 13 and 14 must be attributed to K-3-*O*-diglycosides ($[M–H]^-$ of K has *m/z* 285) with the first unit being a glucose and the second one, a rhamnose moiety. The glycose linking types (1→2; neohesperidose and 1→6; rutinose) can be distinguished on the basis of the value of the ratio Y_0^-/Y_1^- [26]. In the spectra, the ratio is <1 which suggests that the compounds have (1→6) linking; that is, they should be K-3-*O*-rutinosides. Peaks 13 and 14 are most likely K-3-*O*-rutinoside (nicotiflorin) and an isomer, in which perhaps one or more OH groups are shifted. Interestingly, in the mass spectrum of peak 14, an *m/z* 563 ion is observed, which is absent from the spectrum of peak 13. This ion can be explained by a loss of H_2CO from $[M–H]^-$. Unfortunately, the spectral difference does not provide a clue to further unravel the structure of the two isomers. As with *T. pratense* and *T. repens*, MS/MS did not show additional fragmentation, the only exception being MS/MS of *m/z* 285, which caused some additional fragmentation, indicating loss of CH_3, CO, and CO_2.

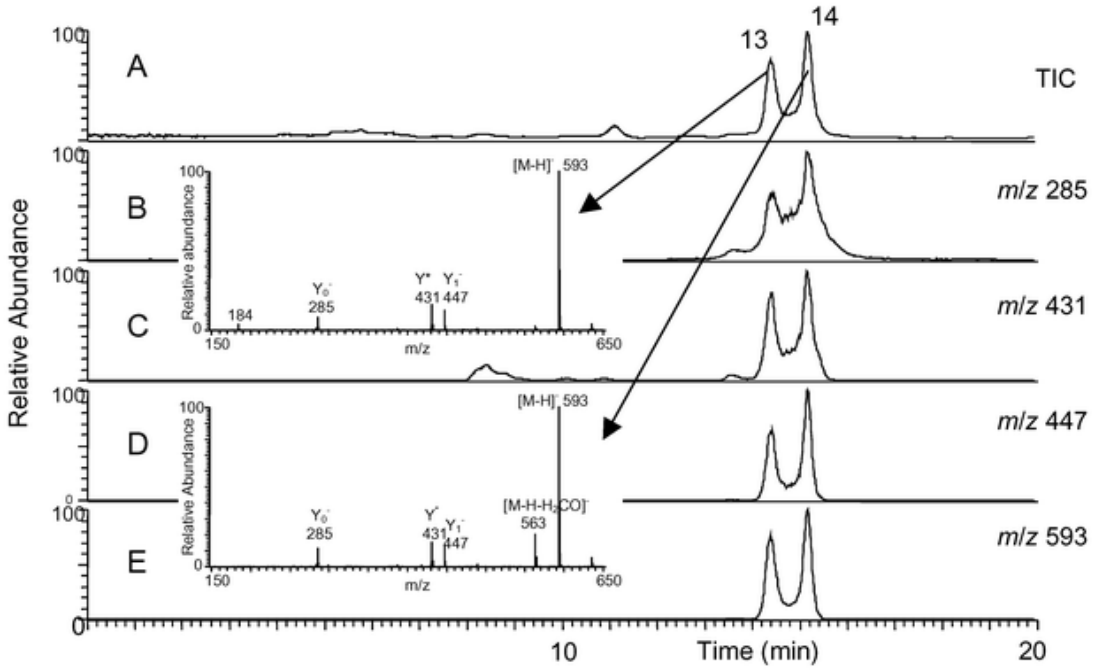


Fig. 7 Full-scan (*A*) and extracted-ion (*B–E*) LC–NI APCI-MS of *L. corniculatus* extract. *Inserts*: mass spectra of peaks 13 and 14.

The two K diglycosides do not seem to occur as part of a satellite set in *L. corniculatus*: no corresponding glycoside–malonates or aglycons were found. The concentrations of peaks 13 and 14 were calculated to be approx. 20 and 30 mg/g of leaves,

respectively, on the basis of the UV_{265} calibration plot of K. That is, *L. corniculatus* displays a substantially higher total flavonoid concentration (ca. 50 mg/g leaves) than *T. pratense* (15 mg/g leaves).

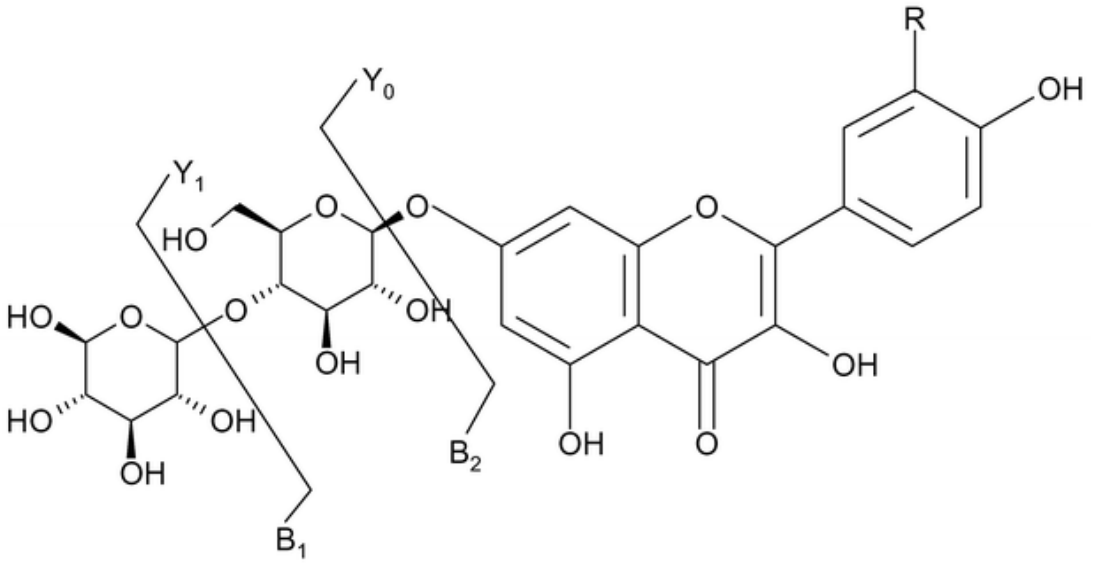


Fig. 8. Carbohydrate ion nomenclature for kaempferol (R=H) and quercetin (R=OH) diglycosides, adapted from [32]. Y denotes the diglycoside, with fragments that contain the flavonoid part of the parent ion being denoted Y_1 (loss of one glycose moiety) and Y_0 (loss of two glycose moieties); in the same way the glycose fragments are denoted B_1 and B_0.

In the literature, several papers deal with the analysis of *L. corniculatus* extracts [28–30], but they were all published some two decades ago. Reynaud et al. [28] isolated and identified several glucosides, rhamnosides, and galactosides of K, Q, corniculatusin, and sexangularetin from leaves using preparative LC and 2D-TLC and identification with UV and comparison with reference samples. Jay et al. [29] isolated and identified the aglycons Q, K, isorhamnetin (Is), fisetin, desoxy-5-K, and geraldol in hydrolyzed leaves extracts, using preparative LC and TLC and identification with UV, off-line MS and NMR. Harborne [30] analyzed *L. corniculatus* flowers and identified quercetagetin, its 3-galactoside and its 7-methyl ether using paper chromatography and spectral identification with shift reagents and comparison with reference samples. Careful screening of our LC–MS results by means of extracted-ion traces did not reveal the Q and K aglycons or any of their methoxy and/or desoxy derivatives mentioned above. Our results partially agree with those of Reynaud et al. [28], who also found K (di)glycosides in the leaves, the difference being that in all cases the glucose and rhamnose groups were in different positions and never linked to each other. None of the quoted papers provided quantitative information.

***T. dubium* L.**

Three main peaks show up in the LC–UV trace of *T. dubium* (Fig. 9). On the basis of the UV absorbance maxima at 255 and 355 nm, peaks 15–17 can be assigned to flavones (cf. Table 2). No peak was detected which displayed native fluorescence. To the best of our knowledge there is only one, somewhat older, paper in which *T. dubium* was analyzed for the presence of flavonoids [19]. No flavonoids were found in that study, but only a stilbene, *trans*-resveratrol, a fungitoxic phytoalexin which is closely related to flavonoids.

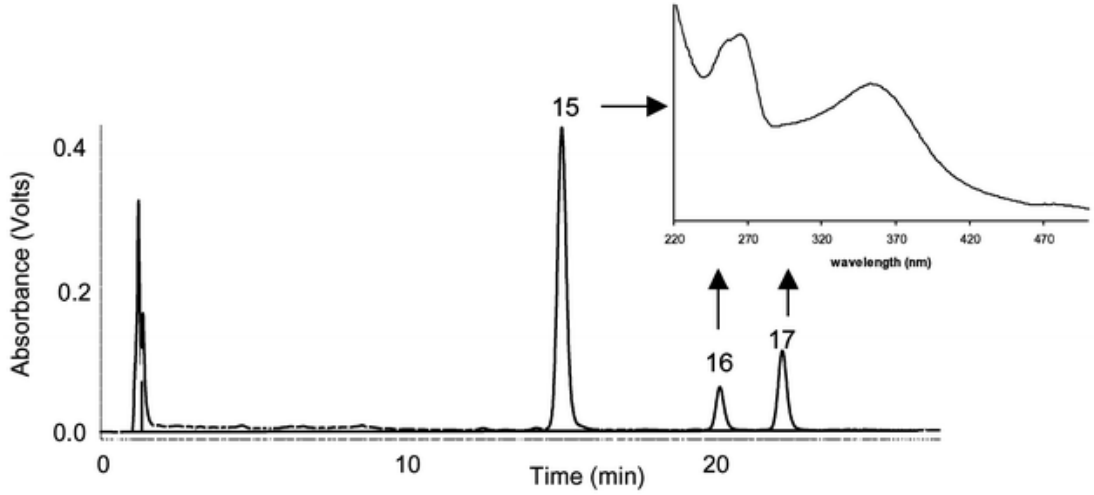


Fig. 9 LC–UV_{265} trace of *T. dubium* extract. Insert: (identical) UV spectra of peaks 15–17.

Fig. 10 shows the full scan and several extracted-ion LC–MS chromatograms of the *T. dubium* extract. Peaks 15–17 obviously are a satellite set; they share the aglycon mass *m/z* 315. Some very distinct fragmentations are observed. Y_1^- (loss of a rhamnose unit) and Y_0^- (loss of a rhamnose and a glucose unit) ions are formed in the case of peak 15, and with peaks 16 and 17 the loss of one glucose unit is observed. Y^* does not show up in any of the spectra, which indicates that the compounds are 7-*O*-flavonols [26]. Compounds 16 and 17 are 7-*O*-glucosides with a single glucose unit, while compound 15 is either a 7-*O*-rutinoside (*O*-rhamnosyl-(α 1→6)-glucose) or a 7-*O*-neohesperidoside (*O*-rhamnosyl-(α 1→2)-glucose). Since $Y_0^-/Y_1^- > 1$, the compound probably has (1→2) linking, i.e., it should be a flavonol-7-*O*-neohesperidoside. The aglycon mass *m/z* 315 most likely can be attributed to Is. This is supported by the observed loss of a CH_3 group and the fact that Is glycosides are among the most common naturally occurring flavonoids [31]. Therefore, compound 15 may well be Is-7-*O*-neohesperidoside, and compounds 16 and 17, Is-7-*O*-glucoside and an isomer with possibly a different position of one or more OH groups or the methyl group. The MS/MS data confirmed that peaks 16 and 17 are isomers and that peaks 15–17 have the same basic structure.

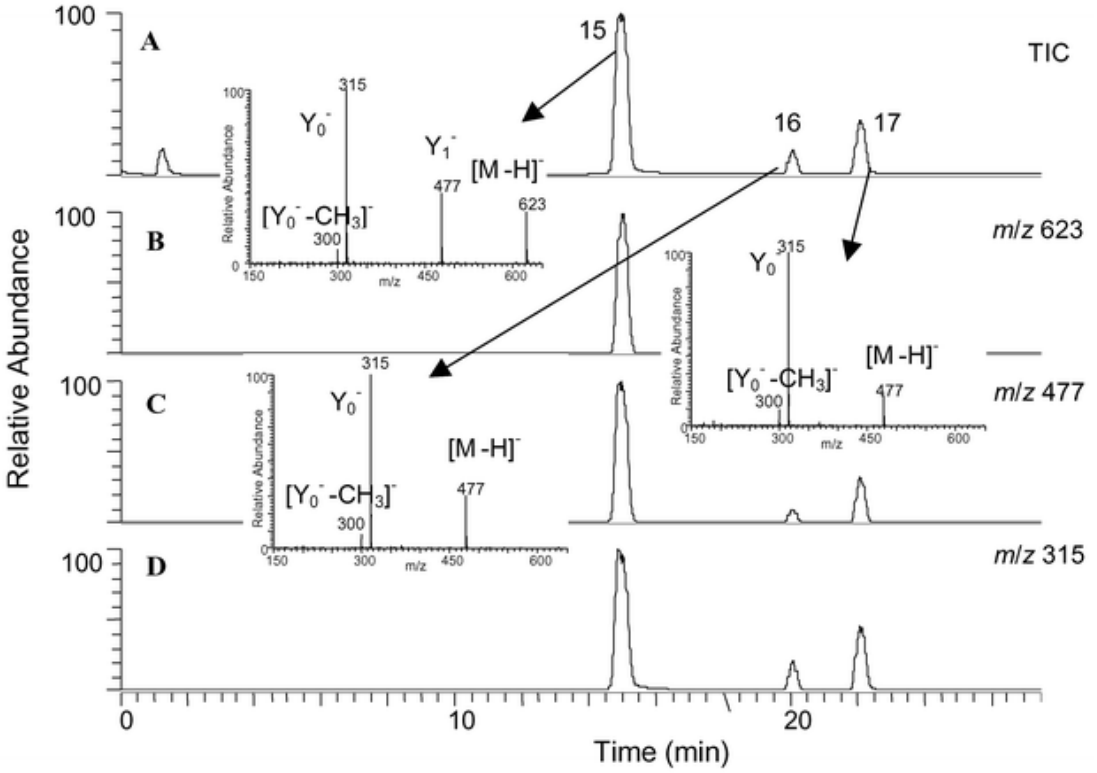


Fig. 10 Full-scan (*A*) and extracted-ion (*B–D*) LC–NI APCI-MS of *T. dubium* extract. *Inserts*: mass spectra of peaks 15–17.

The concentrations of peaks 15–17 were about 50, 5, and 10 mg/g of leaves, respectively; calculations were made by using the UV_{265} calibration plot of Is. This yields a higher total flavonoid content than with any of the other species studied.

5.4 Conclusions

Three LC-based techniques were used to determine the main flavonoids—mainly aglycons, glycosides and diglycosides—in leaves of four species of the Leguminosae family. The detection modes were UV absorbance, fluorescence, and (tandem) mass spectrometry. LC–UV can best be used for a general screening and a first sub-classification and, also, for a semi-quantitative calculation of total flavonoid content. LC–FLU provides only limited additional information because of its, almost too-high, selectivity. It is, however, an excellent tool to identify isoflavones on the basis of their native fluorescence. Most information is derived from full-scan and extracted-ion MS (preferably in the NI APCI mode), which yields characteristic fragmentation patterns of the flavonoids and provides information of the nature of the aglycon, the presence of sugar moieties (e.g., glucose, rhamnose, galactose, rutinose, and neohesperidose), and malonate groups and methyl substituents. MS/MS did not provide much additional information in most instances: generally speaking, the same fragments were

observed as in full-scan MS. For quantification, both LC–MS, in the SIM mode, and LC–UV can be used. Although the former technique provides more selectivity and, often, sensitivity, for the goal of the present study LC–UV was preferred because of the rather similar detectability of most flavonoids and the essentially aglycon-based UV absorption which facilitates quantification because of the limited number of calibration plots that are required.

To the best of our knowledge, this is the first study in which four Leguminosae species have been compared under the same experimental conditions. Several interesting observations are summarized below:

1. The flavonoid patterns of the four plant species are widely different. In *T. pratense* and *T. repens*, the main constituents are flavonoid glucoside–(di)malonates, while *T. dubium* and *L. corniculatus* mainly contain flavonoid (di)glycosides.
2. So-called satellite sets comprising an aglycon, the glucoside and glucoside–malonates or –acetates, are abundantly present in *T. pratense*, where some 13 such sets were identified. Quite differently, at best one satellite set was found in the leaves of the other three plants.
3. The predominant aglycons were F and B in the case of *T. pratense*, but kaempferol in the case of L. *corniculatus*, to mention but one striking difference. Similarly, as regards the sugars, glucose was found to be the most prominent in *T. pratense*, but glucose and rhamnose in the case of *T. dubium*.
4. It is interesting to note that, in several instances, pairs of isomers of, e.g., glucoside–malonates, rutinosides, and neohesperidosides were identified (by MS and MS/MS) in the extracts. Such pairs have also been reported in a few studies in the literature, but little additional information was provided. We are currently investigating the potential of LC–NMR for further structure elucidation.

As regards total flavonoid contents, these showed large mutual differences, from 50–65 mg/g for *L. corniculatus* and *T. dubium*, to 15 mg/g for *T. pratense* and only 1 mg/g for *T. repens*. In the literature, flavonoids in leaves were quantified in only two cases, and both extracts were hydrolyzed before analysis. We found much higher concentrations—15 vs. 5 mg/g for *T. pratense*, and 1 vs. 0.03 mg/g for *T. repens*. This may well be due at least partly to the use of different extraction conditions; the elevated temperatures used for hydrolysis can cause degradation of the target analytes in the extracts. Obviously, if more detailed information than mere aglycon-based quantification is sought, careful attention to sample treatment is required.

One further striking conclusion is that, for all plants except *T. pratense*, our observations markedly differ from those reported in the literature, while the literature results—if more than one is available—also differ from each other. The combined information from the LC–DAD UV runs and all relevant extracted-ion traces makes this abundantly clear. A partial explanation may be differences in environmental factors, growth

conditions, etc. However, no pertinent information is provided in any of the quoted studies; this indicates that further work is required in this area.

5.5 References

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6. Liquid chromatography coupled to nuclear magnetic resonance spectroscopy for the identification of isoflavone–glucoside–malonates in *T. pratense* L. leaves[1].

Summary

Previous studies revealed that the main isoflavones in extracts of leaves of *T. pratense* L. are biochanin A and formononetin, their 7-*O*-glucosides and two glucoside–malonate isomers of each of them. Since LC–MS(/MS) did not provide sufficient information to distinguish the glucoside–malonate isomers, in the present paper LC–NMR as well as off-line two-dimensional NMR was used to obtain further structural information. Matrix solid-phase dispersion (MSPD) was applied to obtain sufficiently high analyte concentrations to perform LC–NMR. Stop-flow reversed-phase LC–NMR was performed using a gradient of deuterated water and deuterated acetonitrile. Off-line COSY and NOESY experiments were carried out to determine the positions of the glucose moiety on the flavonoid aglycone, and of the malonate moiety on the glucose. Based on the fragmentation patterns in MS/MS and the NMR spectra, the two formononetin–glucoside–malonate isomers were identified as 7-*O*-β-D-glucoside–6″-*O*-malonate and 7-*O*-β-D-glucoside–4″-*O*-malonate; *i.e.* they only differ in the substitution position of the malonate group on the glucoside ring. The biochanin A glucoside–malonate isomers, however, have quite different structures. The main and later eluting isomer is biochanin A–7-*O*-β-D-glucoside–6″-*O*-malonate, and the minor and earlier eluting isomer is 5-hydroxy-7-methoxyisoflavone–4′-*O*-β-D-glucoside–4″-*O*-malonate: the positions of the methoxy group and the glucoside–6″-*O*-malonate group on the flavonoid skeleton are interchanged.

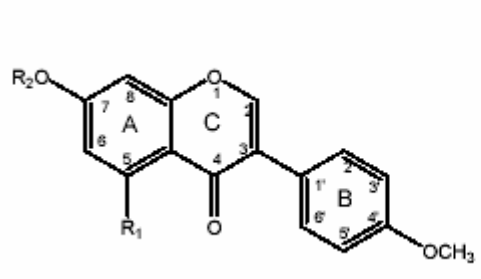
[1]E. de Rijke, F. de Kanter, F. Ariese, U.A. Th. Brinkman and C. Gooijer, *J. Sep. Sci*. 27 (2004) 1061.

6.1 Introduction

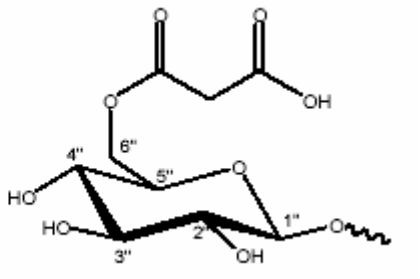
Leguminous plants are known for their rich flavonoid content. In the plant, flavonoids are usually present in their conjugated form, *i.e.* substituted with sugars such as galactose, rhamnose, glucose, neohesperidose and rutinose. The sugars are often further substituted by acyl residues, such as malonate and acetate [1]. In glycosylated form they are accumulated in the vacuole and can serve as pre-formed pools of anti-fungal metabolites that can be mobilized by hydrolysis during infection to release the active aglycones [2]. In species such as alfalfa, soybean and clover, isoflavonoid conjugates also play a role in activating the *nod*-genes of nodulating *Rhizobium* bacteria [3–5].

In many plants of the *Leguminosae*, especially isoflavone–glucoside–malonates are present in quite high concentrations. Formononetin– and biochanin A–glucoside–malonates are the main isoflavone constituents of the roots of *Baptisia australis*, *Ononis spinosa, Trifolium repens*, of the roots and stems of *Trifolium incarnatum* and *Cicer arietinum*, and the roots, stems and leaves of *Trifolium pratense* L.. In *T. pratense* L. (red clover) they constitute about 70% of the pools of formononetin and biochanin A, in *C. arietinum* even up to 90% [6,7]. Also, several glucoside–(di)malonates of anthocyanidins have been identified in *Liliaceae* [8–10], and more recently covalent complexes of an anthocyianidin glycoside and a flavonol glycoside have been found where the compounds share a malonate moiety that is 6″-linked to the cynanidin–glucoside and 3″-linked to the flavonol–glucoside [11].

Table 1. Structures and acronyms of the isoflavones studied.



Isoflavone structure



7-*O*-β-D-glucoside–6”-*O*-malonate

Isoflavone	Abbrev.	R_1	R_2	MW
Formononetin	F	H	H	268
Ononin	FG	H	Glc	430
Formononetin–7-*O*-β-D-glucoside–6”-*O*-malonate	FGM	H	Glc–Mal	516
Biochanin A	B	OH	H	284
Sissotrin	BG	OH	Glc	446
Biochanin A–7-*O*-β-D-glucoside–6”-*O*-malonate	BGM	OH	Glc–Mal	532

In previous papers [12,13] we used reversed-phase liquid chromatography (LC) coupled to atmospheric pressure chemical ionization (tandem) mass spectrometry (APCI-MS(/MS)) and diode-array UV absorbance detection to identify the main isoflavones and their conjugates in *T. pratense* L. (for structures and abbreviation, see Table 1). Only the isomers of the biochanin A– and formononetin–glucoside–malonates could not be unambiguously identified. Therefore, in the present study LC coupled to ^{1}H nuclear magnetic resonance (NMR) spectroscopy is used to obtain further structural information.

Recently, several papers were published on the use of LC–NMR for the on-line identification of flavonoids and related compounds in crude plant extracts. In most papers stop-flow conventional-size LC–NMR is used combined with solvent suppression (*e.g.* [14–16]), while a few papers report on-flow experiments [17,18]. In the latter case, low flow rates are needed to obtain a sufficiently high number of scans per LC peak. If solvent suppression is not sufficient, deuterated solvents should be used. This was the approach followed in our study. In order to obtain the necessary high analyte concentrations, extraction of the freeze-dried *T. pratense* leaves was performed by using matrix solid-phase dispersion (MSPD): it provides both efficient extraction and enables analyte preconcentration.

In a separate set of experiments, LC fractions were collected and off-line COSY (Correlation SpectroscopY) [19] and NOESY (Nuclear Overhauser Enhancement SpectroscopY) [20] NMR spectra of the isolated isomers were recorded to confirm the positions of the glucose and malonate moieties. COSY 2D NMR spectra show correlations (cross-peaks) of scalar (J) coupled pairs of nuclei. Since coupled protons are usually separated by two or three bonds, the connectivity can be derived from the COSY spectrum. In proton NMR, NOESY yields correlations which are caused by dipolar cross-relaxation between protons which are close to one another in space.

6.2 Experimental

Materials

Biochanin A and biochanin A–7-*O*-glucoside were purchased from Indofine (Somerville, NJ, USA), formononetin and formononetin–7-*O*-glucoside from Roth (Karlsruhe, Germany). Acetonitrile, methanol, trifluoroacetic acid and formic acid were from J.T. Baker (Deventer, the Netherlands), white quartz sand and CD_3CN (99.8%) from Aldrich (Steinheim, Germany). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Merck (Darmstadt, Germany) and D_2O (99.9%) from ARC Laboratories (Apeldoorn, the Netherlands). Ultrapure water was

prepared with a Millipore-Academic system (Etten-Leur, the Netherlands). The extracts were prepared from *T. pratense* L. leaves from plants collected in a wetland in the Netherlands (Horstermeerpolder). The plants were grown in a greenhouse. All extracts were filtered over a 0.45 μm filter before injection.

MSPD extraction

Freeze-dried *T. pratense* leaves (0.5 g), 1.5 g of Isolute C18 sorbent and 1 ml of a 350 mM aqueous Tris solution were ground to a fine homogeneous powder. The powder was packed into an SPE column with a polyethylene frit at the bottom and top. The column was washed with 10 ml of distilled water (reddish-brown phase eluted from the column) and the flavonoids were eluted with 5 ml of methanol–water (9:1, *v/v*). The extract was evaporated under a gentle stream of nitrogen and the residue dissolved in 0.5 ml of CD_3CN–D_2O (1:1, *v/v*). This solution was filtered over a 0.2 μm filter and stored at –20°C until use.

LC fractionation

To record off-line COSY and NOESY NMR spectra, 5 g of leaves were extracted. Since such sample amounts cannot be readily handled by MSPD, the extract was prepared as described previously [12]. The *T. pratense* extract was evaporated under nitrogen and re-dissolved in 150 μl methanol–water (1:1, *v/v*). The complete extract (150 μl) was injected onto the LC system, fractions were collected after LC separation (for details, see [12]), the LC eluent was evaporated with nitrogen and the peaks were re-dissolved in CD_3OD–D_2O (1:1, *v/v*).

LC–APCI-MS

For LC–MS a Shimadzu (Princeton, NJ, USA) LC system was used consisting of two LC-10A pumps, a SCL 10A controller unit, a DGU-14A degasser, a SIL-10AD auto-injector and a SPD-10A UV detector (set at 265 and 250 nm). The set-up was coupled to a Thermo Quest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The separation was carried out on a 250 x 4.6 mm I.D. 5 μm Zorbax SB-C_{18} column. LC conditions and MS(/MS) settings are described elsewhere [12]. APCI spectra were obtained in both the negative (NI) and the positive (PI) ionization mode in the *m/z* 150–650 range.

LC–UV–NMR

A Bischoff (Leonberg, Germany) Lambda-1010 UV/Vis detector, a Bruker (Rheinstetten, Germany) LC 22 HPLC pump with a 100 µl injection loop and a BSFU interface were used. The system was controlled by Bruker LC-Hystar v. 1.2 software and a Bruker SCPA Universal Serial Control Interface. The outlet of the BSFU was connected by a PEEK capillary to a 120 µl continuous flow probe. The NMR spectrometer was a Bruker Avance 400 equipped with Bruker XWIN-NMR software for acquisition and processing of the NMR spectra. The LC column was the same as used in LC–APCI-MS, but for detection with NMR the solvents used were (A) CD_3CN and (B) D_2O, both containing 0.1% TFA. Gradient elution of 10% A to 60% A in 50 min, followed by an isocratic step of 60% A over 10 min, was performed at a flow-rate of 1 ml/min. Stop-flow ^1H NMR measurements of the peaks in the chromatograms were governed by the signal of the UV detector, set to 250 nm. The number of transients which were accumulated for the spectra depended on the concentration of the analytes in the sample. The off-line COSY and NOESY spectra were recorded on the same NMR instrument.

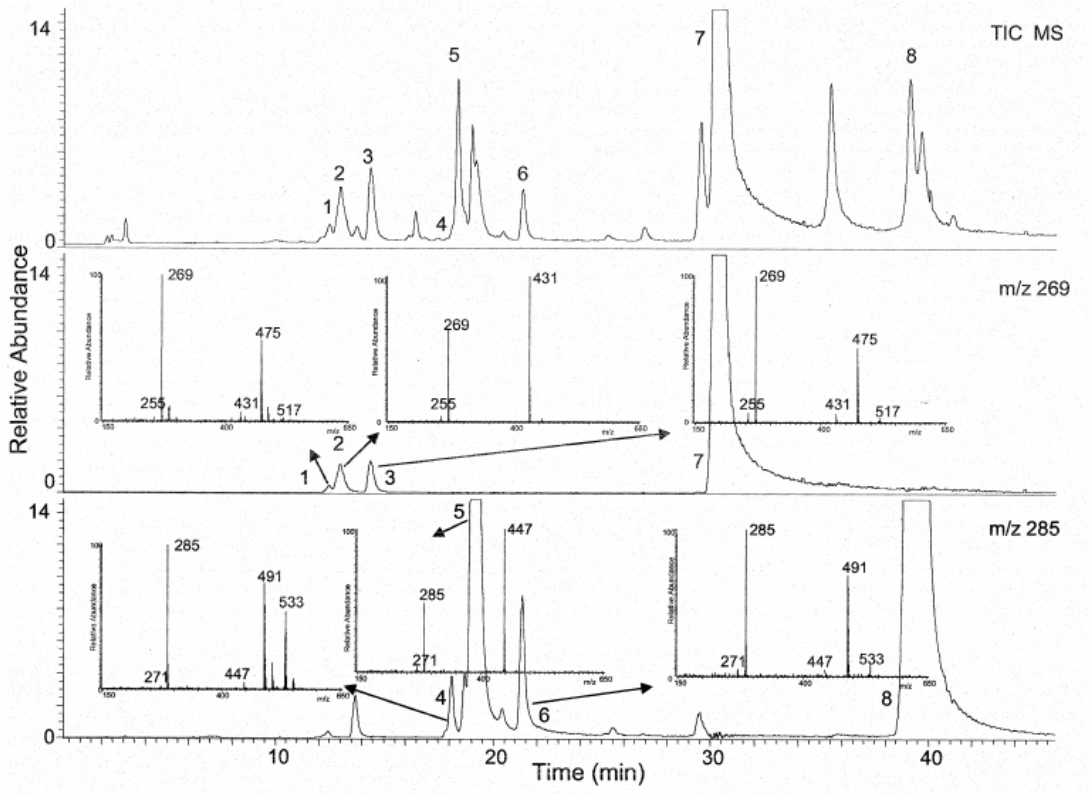


Fig. 1. LC–PI-APCI-MS of *T. pratense* extract. Peak assignment: (1) isomer of peak 3 (FGMi), (2) formononetin–7-*O*-β-D-glucoside (FG), (3) formononetin–7-*O*-β-D-glucoside–6″-*O*-malonate (FGM), (4) isomer of peak 6 (BGMi), (5) biochanin A–7-*O*-β-D-glucoside (BG), (6) biochanin A–7-*O*-β-D-glucoside–6″-*O*-malonate (BGM), (7) formononetin (F), and (8) biochanin A (B). For experimental conditions, see text.

6.3 Results

LC–MS(/MS) and LC–NMR

Since another extraction technique was used than in earlier work, for the LC–NMR experiments the relevant LC–MS(/MS) experiments were repeated. In the current study PI gave more intense mass spectra than NI, presumably since minor amounts of TFA used for the LC–NMR studies were still present in the system and caused ion suppression in NI-APCI. The results are collected in Fig. 1 which shows the full-scan and two extracted-ion LC–MS chromatograms of the *T. pratense* extract. The assignment given in the figure caption is in full agreement with results reported before [12]. Based on the mass spectra and on authentic standards, peaks 2 and 7 were readily identified as FG and F, respectively. In an earlier study [7], hydrolysis of peak 3 resulted in the formation of FG. Based on the mass spectrum and the literature [21,23], peak 3 was identified as FGM, while peak 1 – which had an identical mass spectrum – was assumed to be an unknown isomer, denoted as FGMi. A similar analysis resulted in the identification of peaks 5 and 8 as BG and B, and peaks 4 and 6 as BGM and its unknown isomer, BGMi, respectively. Although a difference is observed in the relative intensity of the *m/z* 533 peak in the spectra of BGM and its isomer, peaks 1 and 3 cannot be unambiguously identified on the basis of their mass spectra; the same holds for peaks 4 and 6. For further confirmation of the proposed structures, MS/MS was performed on the molecular ion and the main fragment ions of each peak. However, the MS/MS spectra did not provide much additional information; they did not show any fragment ions other than the ones observed in full-scan MS. Therefore, LC–NMR was used.

Conventional-size LC–NMR combined with solvent-suppression techniques gave no satisfactory results since the remaining signals of the protonated solvents interfered heavily with the relevant analyte signals. For this reason we used deuterated solvents in combination with solvent suppression. The retention times of peaks 1–8 were slightly different from those in Fig. 1 due to the change in LC conditions needed to apply NMR detection: methanol and ammonium formate were replaced by CD_3CN and D_2O (containing 0.1% *v/v* TFA), respectively. DAD/UV spectra were used for peak assignment. The spectra of peaks 1–3 and 7 are closely similar, confirming that they share the same aglycone; the same is true for peaks 4–6 and 8.

One-dimensional ^1H NMR spectra of peaks 1–8 were recorded with approx. 1052 scans per peak; the associated chemical shifts are listed in Table 2. The NMR spectra of peaks 1–6 all showed signals in the aliphatic region between 3.7–4.1 ppm and at 5.5 ppm, indicating that they come from glucosides. Fig. 2 shows the NMR spectra of peaks 1–3. The five

characteristic aromatic signals between 7.0–9.0 ppm (see also Table 2) confirm that these peaks are conjugates of peak 7 (F). The signals in the NMR spectra were assigned to the various protons indicated in the figure, an assignment based on literature information [23,24]. Furthermore, peaks 1–3 can be attributed to 7-*O*-glucosides because of the signal of the anomeric proton H-1″ at 5.5 ppm and the multiplets between 3.7–4.1 ppm. The anomeric proton was coupled to H-2″ (J = 7.5 Hz), indicating that the compounds are β-D-glucopyranosides [25]. The signal at 4.2 ppm originates from the $-OCH_3$ group. The signal around 4.75 ppm is from residual HDO, while the signal at 3.6 ppm is caused by traces of methanol which was used during sample preparation. The methylene protons of the malonyl residue were not observed due to exchange with the protons of the solvents used [26]. The spectra of peaks 1 and 3 differ from that of peak 2 in the signals that originate from the glucose group; the presence of an acyl group in acylated sugars influences the signals of the sugar group [27]. In the case of peaks 1 and 3 the acyl group is a malonate, an assignment based on the loss of 86 amu in the mass spectra. As regards the two isomers, the spectra for peaks 1 and 3 show only minor differences in the 7–9 ppm region, which reflect the aromatic part of the molecule (except for an impurity in the spectrum of peak 3 at 8.3 ppm). These differences suggest that the structural difference between the FGM isomers lies in the position of the malonate group. For more detailed and unambiguous information, COSY and NOESY experiments were performed (see next section).

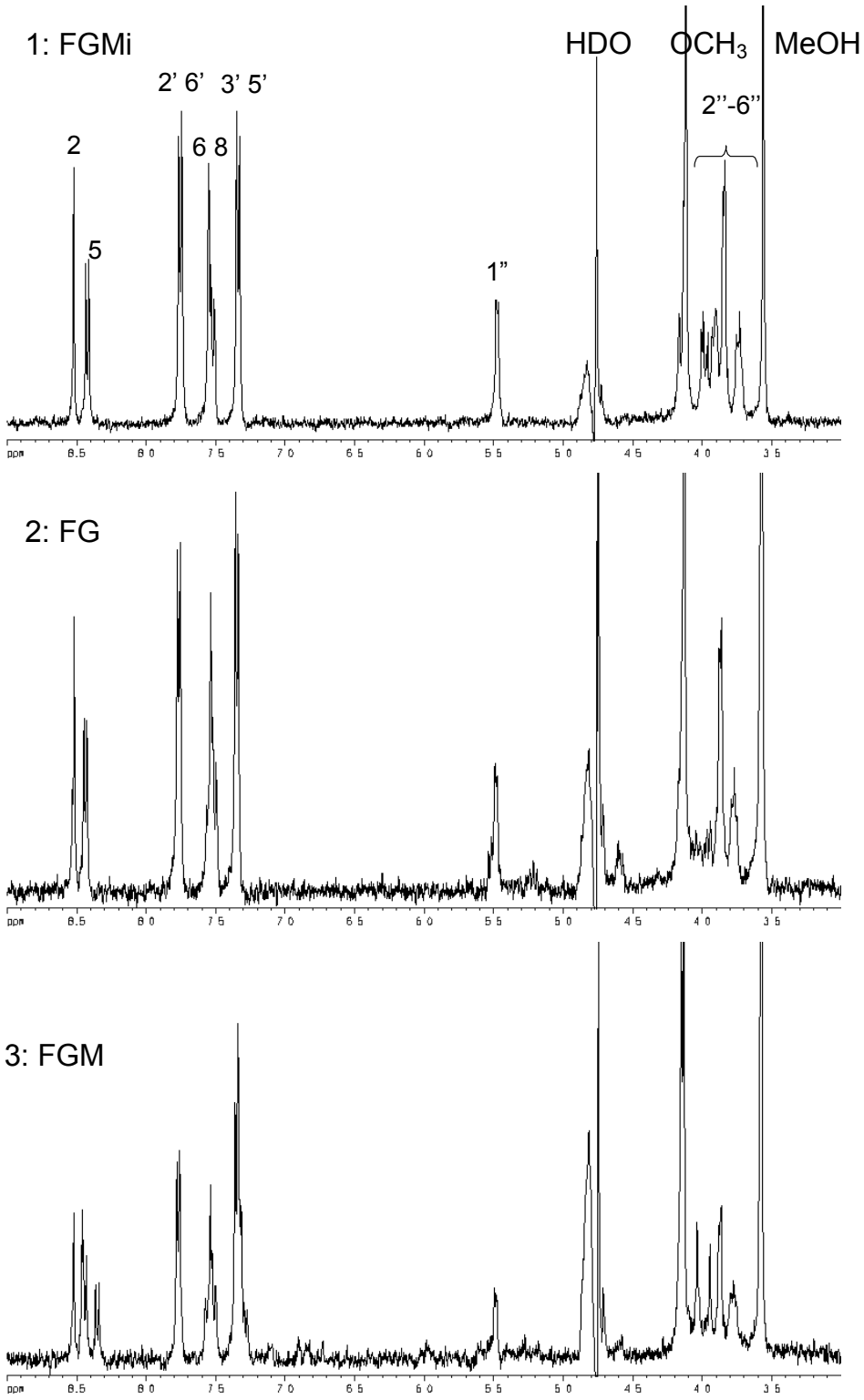


Fig. 2. On-line, stop-flow recorded ^{1}H NMR spectra of peaks FGMi (1), FG (2) and FGM (3). For LC–NMR conditions, see Experimental section.

The on-line NMR spectra of peaks 4–6 are shown in Fig. 3; the chemical shifts are included in Table 2. Also here, signals from $-OCH_3$ (4.2 ppm), residual HDO (4.75 ppm) and traces of methanol (3.6 ppm) are found, and no methylene protons of the malonyl residue. Similar to the results obtained for F, the five characteristic aromatic signals confirm that peaks 4–5 are conjugates of peak 8 (B). Peaks 5 and 6 showed partial overlap in the chromatograms, as can be seen from the small residual signals at 7.4, 7.1 and 6.9 ppm of peak 5 in the spectrum of peak 6. Assignment of the NMR signals to the various protons was based on the literature [23,24]. Contrary to what was observed for the FGM/FGMi pair, for BGM/BGMi the NMR spectra are clearly different in the aromatic region. In the spectrum of peak 6, an upfield shift is observed in the H-8 and H-6 signals from 7.1 to 7.0 ppm and from 6.9 to 6.8 ppm, respectively, as indicated in the figure. Also, the signal originating from H-3′/H-5′ is shifted downfield from 7.4 to 7.5 ppm. These shifts, which indicate differences in the aglycone part of BGM and BGMi, will be discussed in the next section. Analogously to the discussion of Fig. 2, the signal of the anomeric proton H-1″ at 5.5 ppm and the multiplets at 3.7–3.9 ppm indicate that we are dealing with 7-*O*-glucosides, as expected; the coupling of the anomeric proton to H-2″ (J = 7.5 Hz) suggests that the compounds are β-D-glucopyranosides. In the spectra of peaks 4 and 6 – but not peak 5 – signals from the glucose group are observed around 4.0 and 5.3 ppm, respectively, due to the presence of a malonate group on the glucose ring. The aliphatic regions of peaks 4 and 6 are not identical; this indicates that the glucose–malonate group may be in a different position. COSY and NOESY experiments are, again, required to achieve unambiguous structural information.

Table 2. ^{1}H NMR spectral data of *T.pratense* isoflavones with LC–NMR (D_2O–CD_3CN; chemical shift D_2O, 4.75 ppm).

Flavonoid	**Aglycone part**						**a.p.**	**OCH_3**	**Glc**
	2	5	2′ and 6′	3′ and 5′	6	8	1″		2″-6″
FGMi	8.5 (s)	8.4 (d)	7.8 (d)	7.4 (s)	7.6 (d)		5.5 (d)	4.2 (s)	3.7-4.1 (m)
FG	8.5 (s)	8.4 (d)	7.8 (d)	7.4 (s)	7.6 (d)		5.5 (d)	4.2 (s)	3.7-4.1 (m)
FGM	8.5 (s)	8.4 (d)	7.8 (d)	7.4 (s)	7.6 (d)		5.5 (d)	4.2 (s)	3.7-4.1 (m)
F	8.5 (s)	8.4 (d)	7.8 (d)	7.4 (s)	7.6 (d)			4.2 (s)	
BGMi	8.5 (s)		7.8 (d)	7.3 (d)	6.8 (s)	7.0 (s)	5.5 (d)	4.2 (s)	3.7-4.1 (m)
BG	8.5 (s)		7.8 (d)	7.4 (d)	6.9 (s)	7.1 (s)	5.5 (d)	4.2 (s)	3.7-4.1 (m)
BGM	8.5 (s)		7.8 (d)	7.4 (d)	6.9 (s)	7.1 (s)	5.5 (d)	4.2 (s)	3.7-4.1 (m)
B	8.5 (s)		7.8 (d)	7.4 (d)	6.9 (s)	7.1 (s)		4.2 (s)	

a) a.p.: anomeric proton, s: singlet, d: doublet, m: multiplet.
b) in bold: deviating shifts of BGMi aglycone protons caused by different positions of methoxy and glucose moieties compared to BGM.

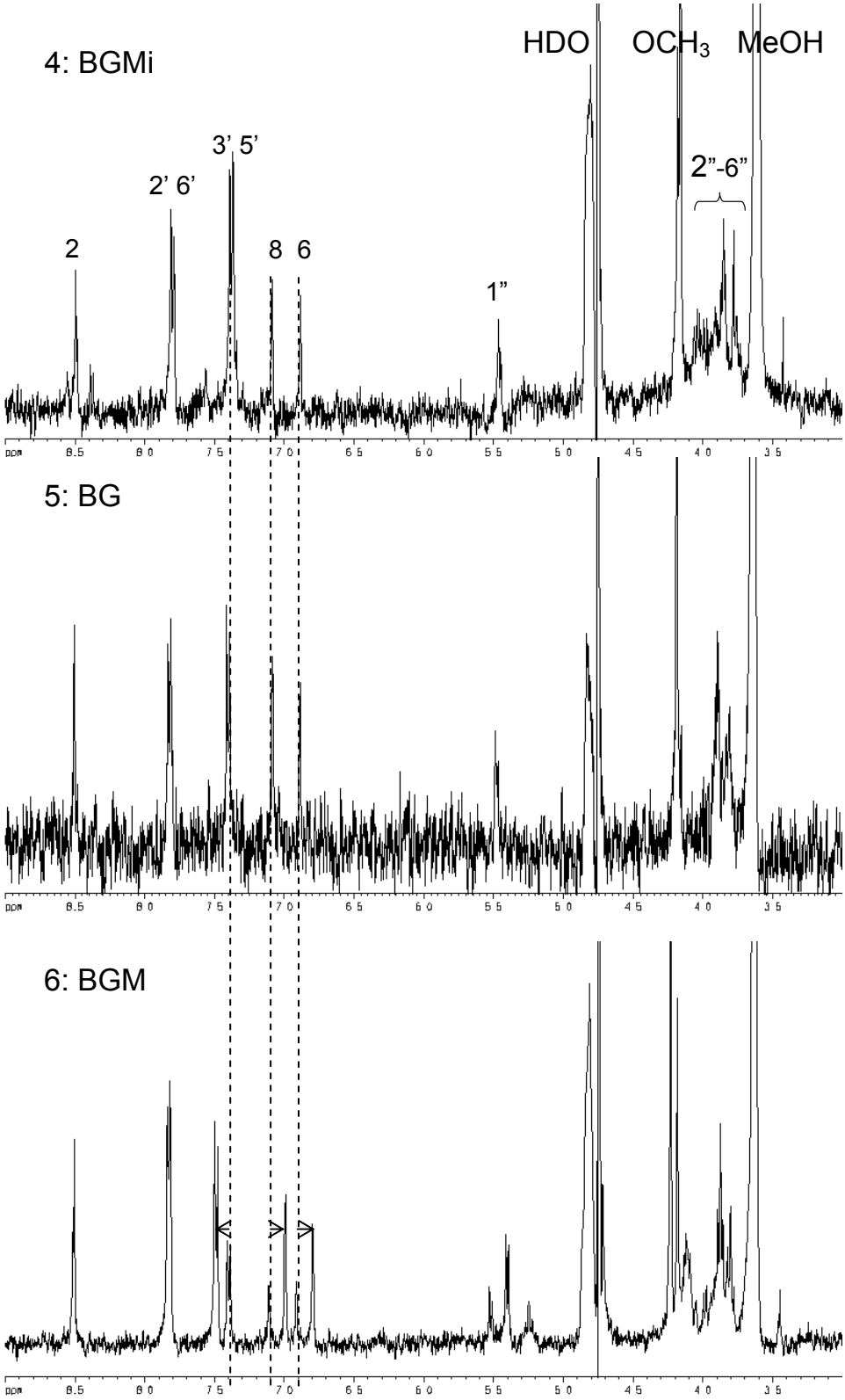


Fig. 3. On-line, stop-flow recorded ^{1}H NMR spectra of peaks BGMi (4), BG (5) and BGM (6). For LC–NMR conditions, see Experimental section.

COSY and NOESY experiments

In order to be able to increase the measurement time for the recording of COSY and NOESY spectra of the glucoside–malonate isomers, these spectra were recorded off-line, using isolated LC fractions. Firstly, off-line one-dimensional ^{1}H spectra of FGMi and FGM were compared (Fig. 4) which provide more details than the spectra of Fig. 2; the spectra of Fig. 2 were recorded in 1 hour during stop-flow LC, while for the spectra of Fig. 4 the scantime was approx. 24 hrs and a much higher concentration was used. As expected, the aromatic parts (7.0–9.0 ppm) of FGMi and FGM in Fig. 4 are fully identical, while there are distinct differences in the glucose region. The signals of the H-6″ protons of the glucose group in the FGM spectrum are shifted to lower field (δH-6″ = 4.35; 4.50), whereas in the FGMi spectrum this holds for the H-4″ proton (δH-4″ = 4.92). This indicates that in FGMi the malonyl group is in the 4″ position and in FGM, the 6″ position. The above assignment of the glucose protons was based on COSY spectra. Similar shifts as observed for the H-4″ and H6″ protons were reported in the literature for the 6″-*O*-malonate of apigenin-7-*O*-glucoside [26] and for the 4″- and 6″-*O*-malonates of the anthocyanin pelargonidin–5-*O*-glucoside [28]. The spectrum of FGM is not completely pure; it also contains signals of FG (*e.g.* the signals of the H-6″ proton at 3.75 and 3.90 ppm) because during the isolation of the FGM peak, a small part of the FG peak was also collected. The peak at 5.00 ppm in the FGMi spectrum is a spike and should be discarded. The NOESY spectra of FGM and of FGMi (not shown) demonstrated correlations between the $–OCH_3$ protons and the H-3′ and H-5′ protons, and between the anomeric proton H-1″ (δH-1″ = 5.27) and the aglycone protons H-6 and H-8. This confirms that in both isomers the methoxy group is in the 4′ position, and the glucose group in the 7-position of the aglycone. Interestingly, we observed that at room temperature in the acidic CD_3OD/D_2O solution FGMi is not stable but partly undergoes an intramolecular rearrangement into FGM in the course of several hours. According to the literature [29] this rearrangement has a reasonable stereochemical basis, takes place easily in an acid environment and is well established. To the best of our knowledge, such a rearrangement has not been observed before for flavonoid–glucoside–malonates.

In the NOESY spectrum of BGM (not shown) correlations were observed between the protons of $–OCH_3$ and the aglycone protons H-3′ and H-5′, and between the anomeric proton H-1′ and the aglycone protons H-6 and H-8, in agreement with the proposed structure in which the methoxy group is in the 4′, and the glucose group in the 7, position of the aglycone [23]. The signals corresponding to the H-6″ of the glucose moiety are shifted downfield to 4.41 and 4.24 ppm, indicating that the malonate is in the 6″ position [26]. The NOESY spectrum of BGMi (Fig. 5), however, shows correlations between the anomeric proton H-1″ and the H-3′ and H-5′ protons of the aglycone, and between the protons of –

OCH_3 and the aglycone protons H-6 and H-8. This demonstrates that, compared to BGM, the glucoside–malonate group and the –OCH_3 group are interchanged: the former now is in the 4′ position, and the latter in the 7 position of the aglycone. This is in line with the upfield shift observed above for the H-6 and H-8 aglycone protons and the downfield shift of the H-3′/H-5′ signal in the spectrum of BGMi. The ^{1}H and COSY spectra (not shown) of BGMi showed that the signal of H-4″ is shifted to lower field (δH-4″ = 5.48), as was also observed for FGMi. This indicates that also in BGMi the malonyl group is in the 4″ position.

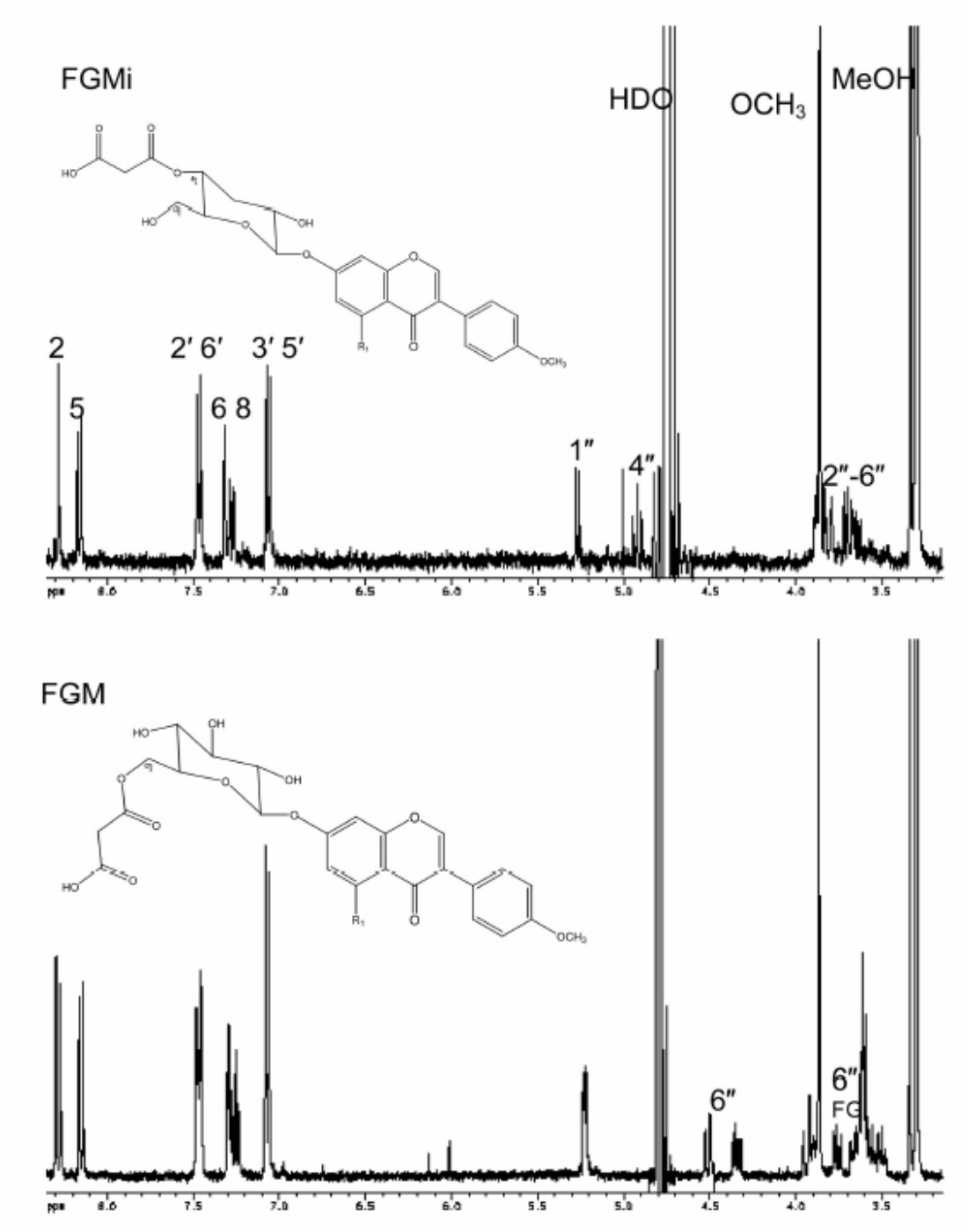


Fig. 4. Off-line recorded ^{1}H NMR spectra of FGMi and FGM in CD_3OD-D_2O (1:1, *v/v*).

As with FGMi, at room temperature BGMi undergoes an intramolecular rearrangement which causes the malonyl group to shift to the 6″ position. Of course, no rearrangement of the 4′ and 7 substituents was observed.

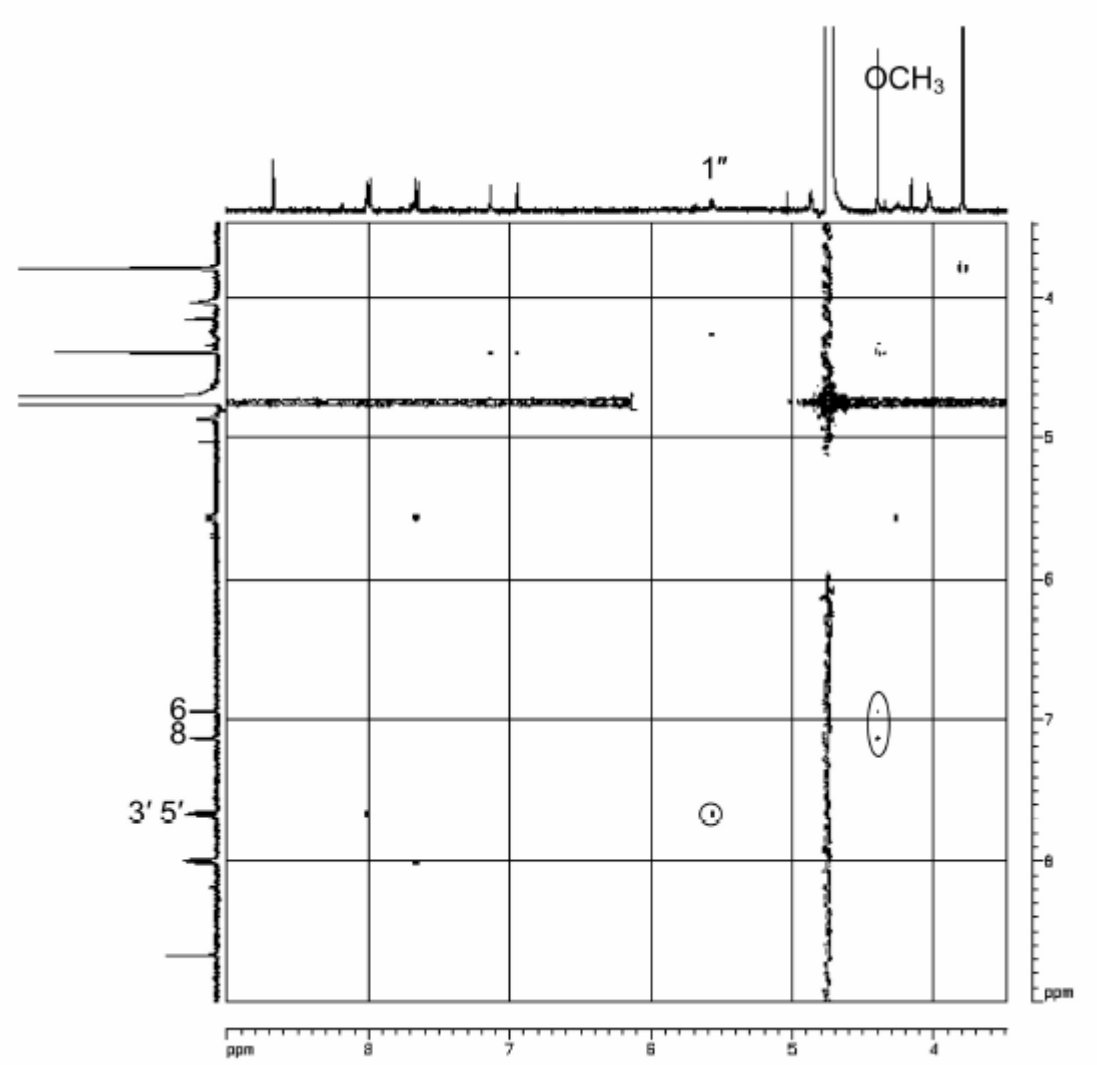


Fig. 5. Off-line recorded NOESY spectrum of BGMi in CD_3OD–D_2O (1:1, *v/v*). The correlations discussed in the text are indicated.

6.4 Discussion and conclusions

The F and B conjugates in *T. pratense* are known [12] to occur as satellite sets of aglycones, glucosides and glucoside–malonates. In the literature, some ambiguity exists about the elution order and relative intensities of the glucoside–malonates isomers [21,22]. Lin *et al.* [21] found two glucoside–malonate isomers for both F and B, and in both cases identified the higher and earlier eluting one as the 6″-*O*-malonate; the structures of the other isomers were not given. They based their identification on an early paper by Beck *et al.* [23], who isolated the F and B glucoside–malonates from *T. pratense* and determined the position of the malonate by

periodate oxidation and subsequent NMR. Klejdus *et al.* [22] found three glucoside–malonate isomers in case of F, and only one glucoside–malonate of B. They reported that for both the highest and last eluting F–glucoside–malonate and for the B–glucoside–malonate, the malonate is in the 6″ position, while they did not assign the malonate positions of the other two F–glucoside–malonate isomers. For the assignment of the 6″-*O*-malonates they refer to a book chapter [30], without providing more details. In the present study, the 6″-*O*-malonates are the higher and later eluting peaks of the satellite sets in line with the assignment of Klejdus *et al.* in the case of F. Most likely the differences in elution order are caused by differences in eluent conditions [13]. Intramolecular rearrangement of the malonyl group after isolation of the individual peaks was not reported in the quoted studies.

As regards the relative intensities of the peaks in a satellite set, in our previous analyses [12], the F and B glucoside–malonate peaks were the highest ones, in line with the observations in ref. [21]. However, in the present study the glucosides were most prominent in the UV trace, as also reported in ref. [22]. Most likely this difference is caused by the extraction conditions: Lin *et al.* [21] dried the leaves and performed the extraction at room temperature, while Klejdus *et al.* [22] used a somewhat higher temperature (30°C), which will promote hydrolysis of the glucoside–malonates. In the current LC–NMR study, the MSPD-based sample preparation was somewhat more elaborate than the methanolic extraction used earlier. Probably, therefore, hydrolysis of the glucoside–malonates was stronger in the former case, causing the glucosides to become the largest peaks. Despite the discrepancies in the quoted studies, there is one consistency: in all instances the 6″-*O*-malonate isomers are the most prominent.

To summarize, by using a combination of LC–MS, LC–NMR and off-line 2D NMR, the FGM and BGM isomers found in red clover were unambiguously identified. The isomers are –in order of elution– formononetin–7-*O*-β-D-glucoside–4″-*O*-malonate, formononetin–7-*O*-β-D-glucoside–6″-*O*-malonate, 5-hydroxy-7-methoxyisoflavone–4′-*O*-glucoside–4″-*O*-malonate and biochanin A–7-*O*-β-D-glucoside–6″-*O*-malonate.

Acknowledgements

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7. Changed isoflavone levels in red clover (*Trifolium pratense* L.) leaves with disturbed root nodulation in response to waterlogging[1]

Summary

The effect of disturbed root nodulation on the quantitative and qualitative composition of the main isoflavonoid glucoside malonates, glucosides and free aglycones in the leaves of *Trifolium pratense* L. grown under waterlogging conditions was investigated. Isoflavonoids are involved in the regulation of root nodule activity and the establishment of the mycorrhizal association. Isoflavonoid determination was done using reversed-phase liquid chromatography coupled to mass spectrometric and UV absorbance detection. It was found that in response to waterlogging the concentrations of the free aglycone biochanin A and the conjugates biochanin A–7-*O*-glucoside–malonate, biochanin A–7-*O*-glucoside, and gensitein–7-*O*-glucoside in the leaves increased 2–3-fold after a lag period of three weeks, due to disturbed root nodulation. The other isoflavones detected — the free aglycone formononetin, and the conjugates formononetin–7-*O*-glucoside–malonate and formononetin–7-*O*-glucoside — did not show any significant changes related to waterlogging. After restoring the normal soil water conditions, the concentrations of biochanin A and its two conjugates rapidly returned to the initial values, while the concentration of genistein–7-*O*-glucoside remained high.

[1]E. de Rijke, L. Aardenburg, J. van Dijk, F. Ariese, W.H. O. Ernst, C. Gooijer, U.A.Th. Brinkman, *J.Chem Ecol.* 31 (2005) 1237.

7.1 Introduction

Because of the importance of wetlands for the conservation of biodiversity, the restoration of wetlands is a goal in many countries worldwide. Restoration activities include changes in grassland management and re-wetting dry (polder) areas, and can have great impact on ecological processes, especially affecting those plant species that are sensitive to waterlogging and/or flooding. Flood-resistant plants can easily acclimatize to wet conditions, but in non-flood-resistant species flooding may cause metabolic problems due to oxygen deficiency and enhanced availability of reduced metals [1].

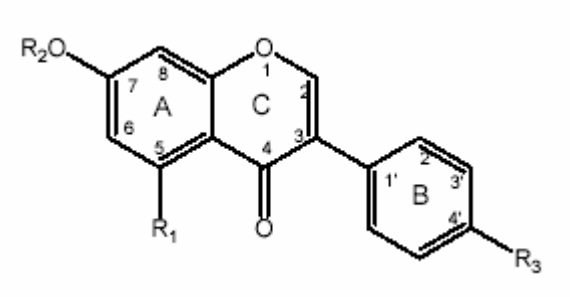
Red clover (*Trifolium pratense*, Leguminosea) is a non-flood-resistant species that commonly occurs in moist grasslands. Trifolium species are known symbiotic nitrogen fixating crops that are used in sustainable agriculture to restore N fertility of a soil [2,3]. In symbiotic plant-rhizobium interactions flavonoids play an important role as signal transducing compounds. The isoflavonoids, for example, are involved as nodulation inducers [4]. In a variety of leguminous plants they can influence the early stages of the establishment of biological nitrogen fixation in root nodules after infection by *Rhizobium* bacteria [5].

In *T. pratense* over 40 flavonoids were identified [6–8]. They constitute one of the most important groups of natural products found in plants – in total more than 6000 flavonoids have been identified and documented [9]. In plants they are usually present as glycosides, *i.e.* provided with one or more sugar groups such as glucose, galactose or rhamnose. The sugars are often further substituted by acyl residues, such as malonate, acetate, 4-coumarate, caffeate or ferulate [10]. Glycosylated compounds, rather than free aglycones, are accumulated in plants; they are better stored within plant vacuoles and are less reactive towards other cellular components than aglycones. They are therefore often thought of as detoxification products or as physiologically inactive plant storage forms [10]. The flavonoid family comprises several subclasses such as flavones, flavonols, flavanones and isoflavones. From a physiological point of view, flavonoids are of interest because of their various biological activities: as screen against elevated UV-B radiation [11–13], infection by microorganisms [14], herbivore attack [15], and water stress [16,17]. Flavonoids – and isoflavonoids in particular – are also involved in the induction of the expression of *Rhizobium* nodulation genes from leguminous plants [18,19].

The aim of this study was to investigate the effect of disturbed root nodulation on the concentrations of the main isoflavones of *T. pratense*, including aglycones, glucosides and glucoside malonates. To that means, *T. pratense* plants were subjected to two different soil water regimes to study the effect of disturbed root nodulation in response to waterlogging on the levels of their main isoflavonoids (for structures and abbreviations, see Table 1). The study focusses on isoflavonoids in the leaves because the isoflavonoids are known to respond

to environmental disturbances [4] and the leaves of *T. pratense* have the highest concentrations. In roots the flavonoid concentrations are much lower and furthermore, less root mass is available for analysis. To study the isoflavonoid profiles and their changes due to environmental effects a very sensitive analysis method is needed. Therefore, a previously reported reversed-phase liquid chromatographic (RPLC) procedure was used, coupled to negative atmospheric pressure chemical ionization mass spectrometry (NI APCI-MS) and UV absorbance detection [7,8].

Table 1. Structures and acronyms of the isoflavones studied.



Isoflavone	Abbrev.	R_1	R_2	R_3	MW
Formononetin	F	H	H	OCH_3	268
Ononin	FG	H	7-*O*-β-D-Glc	OCH_3	430
Formononetin–7-*O*-glucoside–malonate	FGM	H	7-*O*-β-D-Glc–Mal	OCH_3	516
Genistin	GG	OH	7-*O*-β-D-Glc	H	432
Biochanin A	B	OH	H	OCH_3	284
Sissotrin	BG	OH	7-*O*-β-D-Glc	OCH_3	446
Biochanin A–7-*O*-glucoside–malonate	BGM	OH	7-*O*-β-D-Glc–Mal	OCH_3	532

7.2 Materials and methods

Flooding studies

Flooding experiments were performed using *T. pratense* plants grown from commercially obtained seeds (Duchefa, Haarlem, the Netherlands). Seedlings were grown in a greenhouse for four months before starting the experiment. Plants were grown in a controlled-environment greenhouse maintained at 25/20°C day/night temperatures and 60–70% relative humidity, to prevent isoflavonoid concentrations from being affected by changes in environmental conditions. The light conditions during the experiment were those of the natural glass-filtered (UV B) radiation in the period from 1 May to 24 June. The plants were placed in PVC cylinders filled with potting compost containing 22 wt.% organic material (Jongkind, Aalsmeer, the Netherlands) and a wire mesh bottom. The cylinders were placed in plastic pots to enable soil water management. As sampling and extraction can affect flavonoid

concentrations in plants [7], sampling was considered destructive. Therefore, ten separate *T. pratense* plants were placed in each pot. At each sampling date, fresh plants were sampled. Plants were removed from the experiment after sampling. Because all plants in one pot were subjected to the same environmental conditions, they are considered as one block that is sampled repeatedly over time.

The experimental set-up followed a single-factor ANOVA (analysis of variance) design with two levels, waterlogged (W) and normal (N) conditions, and 7 replicates per level. At t = 1 day, the soil water level of the W plants was raised to ensure complete waterlogging of the soil, *i.e.* that the water reaches the soil surface. Throughout the study, the N plants were watered daily to field capacity. Plants were sampled at t = 0, 7, 14, 21, 35 and 42 days and the flavonoid composition of their leaves was determined as described below. At t = 35 days, the soil water level of the W plants was changed to the level of the N plants, and kept at this level until the end of the study.

Materials

Biochanin A and biochanin A–7-*O*-glucoside were purchased from Indofine (Somerville, NJ, USA), and genistein–7-*O*-glucoside, formononetin and formononetin–7-*O*-glucoside from Roth (Karlsruhe, Germany). Methanol and formic acid were from J.T. Baker (Deventer, the Netherlands), ammonium formate and white quartz sand from Aldrich (Steinheim, Germany). Tris(hydroxymethyl)aminomethane (TRIS) was purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, the Netherlands).

Extraction

Extraction of the *T. pratense* leaves was performed as described earlier [20]. Fresh leaves (1 g) were ground with 3 ml of aqueous 350 mM TRIS solution, pH 7.9, for 2 min. One gram of white quartz sand and 10 ml of methanol–water (9:1, *v/v*) were added, and the extract was stirred for 1 min. The extract was filtered over a Büchner funnel and 10 ml of methanol–water (9:1, *v/v*) were added to the residue. The procedure was repeated once and the combined extracts were filtered over a 0.45 μm filter and stored at –20˚C until use. Under these conditions, the extracts are stable for at least two weeks [7]. However, the extraction must be performed as quickly as possible (*i.e.* within max. 10 min) because flavonoid–glucosides in freshly picked leaves undergo hydrolysis to their aglycones [7]. TRIS solution, which functions as a β-glycosidase inhibitor, was added to prevent this hydrolysis.

Instrumentation

LC–DAD UV. LC was performed on a HP 1050 LC system (Hewlett Packard, Waldbronn, Germany) with a photo diode array UV detector (DAD), set at 250 nm. For separation a 250x4.6 mm I.D. 5 μm Zorbax SB-C18 LC column was used, with a methanol–10 mM ammonium formate buffer (pH 4.0) gradient at a flow rate of 1.0 ml/min (see [20]). Before use all solvents were filtered over a 0.45 μm filter and degassed with helium. Sample injection (10 μl) was performed with a Midas autosampler (Spark Instruments, Emmen, the Netherlands).

LC–APCI-MS. For LC–MS a Shimadzu (Princeton, NJ, USA) LC system was used consisting of two LC-10A pumps, a SCL 10A controller unit, a DGU-14A degasser, a SIL-10AD auto-injector and a SPD-10A UV detector (set at 250 nm). The autosampler was cooled to prevent hydrolysis of flavonoid glucoside–malonates in the extracts. The set-up was coupled to a Thermo Quest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The injection volume, column and gradients were the same as in LC–DAD UV. APCI spectra were obtained in the negative ionization mode in the *m/z* 150–650 range. The capillary and vaporizer temperatures were 250˚C and 450˚C, respectively. The capillary voltage was set at –12 V and the corona discharge current was 10 μA (see [20]). The sheath-gas (nitrogen) flow rate was approx. 200 l/h, and the auxillary gas (nitrogen) flow rate, approx. 50 l/h. The LC flow was directed to the mass spectrometer without stream splitting.

Statistical data analysis

A single-factor repeated-measures ANOVA was performed to test for the effects of soil water level, time of exposure and their possible interaction on plant isoflavone concentrations, *i.e*. to test whether soil water level and time of exposure are independent parameters. Data requirements for the multivariate analysis concerning equality of the covariance matrix were checked using Box's M test. To test whether isoflavone concentrations decreased to the level of the control treatment after restoring the original soil moisture conditions, isoflavone concentrations of both treatments were compared at t = 42 days using an independent samples t-test. Data were checked for normality using Shapiro-Wilk's test. All analyses were performed using the program SPSS version 10.1.0.

7.3 Results

LC–UV/APCI-MS

Fig. 1 shows typical LC–UV and LC–MS chromatograms of an extract of *T. pratense* leaves, which will be discussed below. The advantage of an ion-trap MS instrument is that full-scan (FS) and time-scheduled selected ion monitoring (TS SIM) can be performed simultaneously. FS is used to scan the extracts and TS SIM for quantification of selected (see below) isoflavones. To select the masses to be used in SIM, a 10 mg/l standard mixture of five isoflavone aglycones and glucosides (formononetin–7-*O*-glucoside (FG), biochanin A–7-*O*-glucoside (BG), genistein–7-*O*-glucoside (GG), formononetin (F) and biochanin A (B); *cf.* Table 1) was analyzed in the FS MS mode (*m/z* 150–650). This enabled us to attribute five of the main peaks that show up in Fig. 1 to FG, BG, GG, F and B, based on their mass spectra and retention times. In addition, the strong peaks at 25 and 32 min can be attributed to formononetin–7-*O*-glucoside–malonate (FGM) and biochanin A–7-*O*-glucoside–malonate (BGM), respectively [20]. The compounds were identified based on their aglycone mass and their characteristic fragmentation pattern, using TS SIM (Fig. 1B–D) and FS chromatograms (Fig. 1A), respectively. The main ions that were generated in NI APCI-MS were the pseudo-molecular ions $[M–H]^-$, formic acid adducts $[M+45]^-$, $[M–Glc]^-$, and, when the analytes were glucoside–malonates, $[M–Mal]^-$ and $[M–Glc–Mal]^-$ ions. In all cases, the aglycone ions were selected for TS SIM because they had the highest intensities with the MS settings used.

In a previous paper [8] four ionization modes (PI and NI APCI; PI and NI ESI) were compared using a *T. pratense* extract and optimized LC conditions. In the PI modes, background signals were higher than in the NI modes. Overall, the analyte responses were best in the NI APCI modes. In the mass spectra, differences in relative intensities of the fragment ions were observed depending on the ionization mode and eluent composition.

Analyte detectability was the same for the simultaneous FS–TS SIM method and the conventional TS SIM method, while the gain in sensitivity of TS SIM compared with FS was about 100-fold. Detection limits (LODs; $S/N = 3$) in the TS SIM mode were found to be 1–45 µg/l (R^2 values of calibration plots, 0.993–0.999 in all cases). Because no standards of FGM and BGM – the malonates of FG and BG – are available, their concentrations were calculated, based on the calibration curves of FG and BG, assuming equal MS response of the aglycone ions in SIM. With UV detection, LODs were higher than in TS SIM MS (40–530 µg/l; R^2 values 0.998–0.999); also for that reason, TS SIM MS was used to identify and quantify the isoflavone aglycone, glucoside and acylated glucoside peaks in the extracts. The simultaneous FS and TS SIM method was used throughout the study.

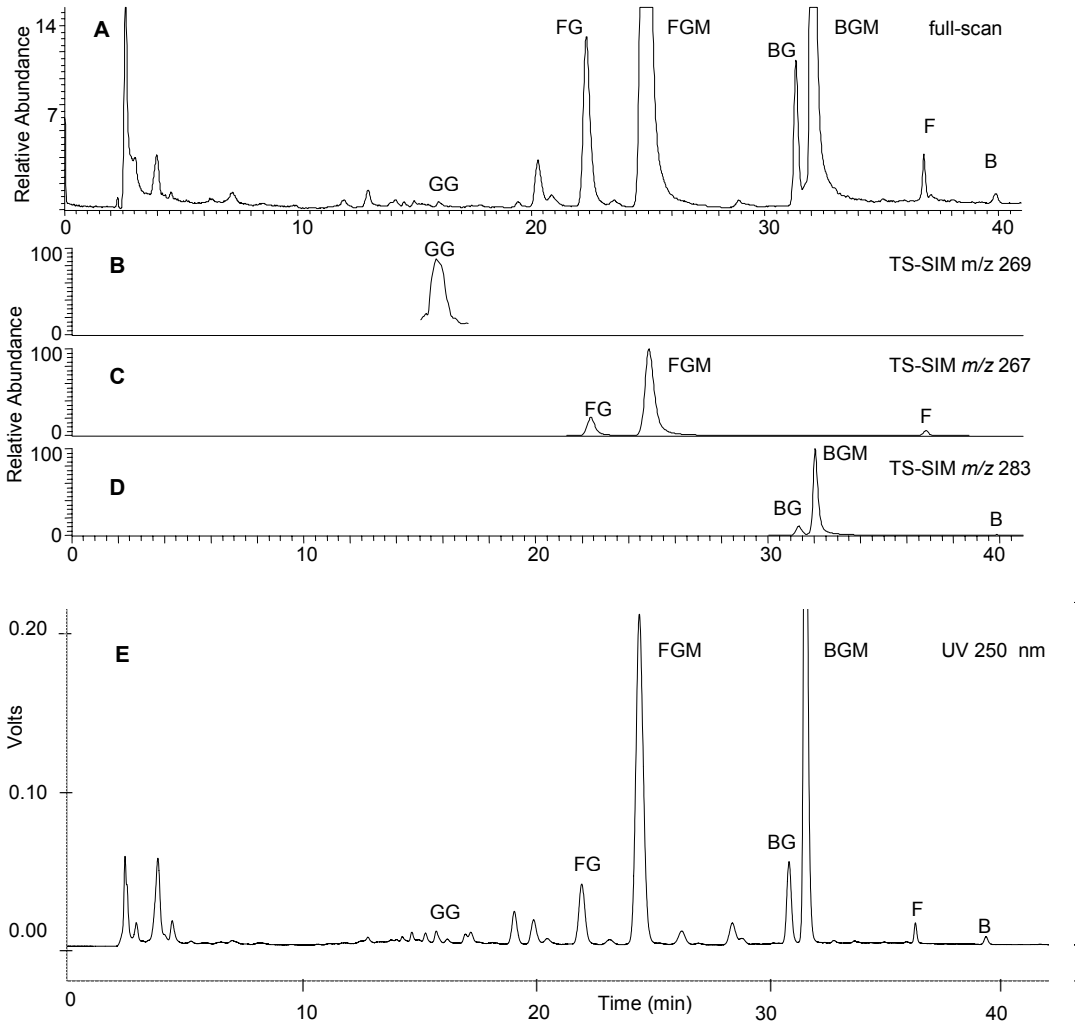


Fig. 1. Reversed-phase LC with (A) full-scan and (B-D) TS-SIM (*m/z* 269 for GG, *m/z* 267 for F, FG, FGM and *m/z* 283 for B, BG, BGM) NI APCI-MS, and (E) UV_{250} of a *T. pratense* extract, recorded at t = 35 days. For details, see text.

Flooding studies

The leaves and roots of the two series of full-grown plants under N and W conditions were visually inspected and compared during the experiment. After three weeks, the leaves of the W plants were slightly smaller than the leaves of the N plants, and some leaves of the W plants had turned yellow. We also observed that the roots of the W plants were gradually reaching the soil surface and that the amount of root nodules on the submerged roots of the W plants was decreasing over the course of the study (Table 2). After 35 days, an approx. two-fold decrease was observed. And at t = 42 days the root nodule level was approx. restored to its initial value. The N plants did not show any changes.

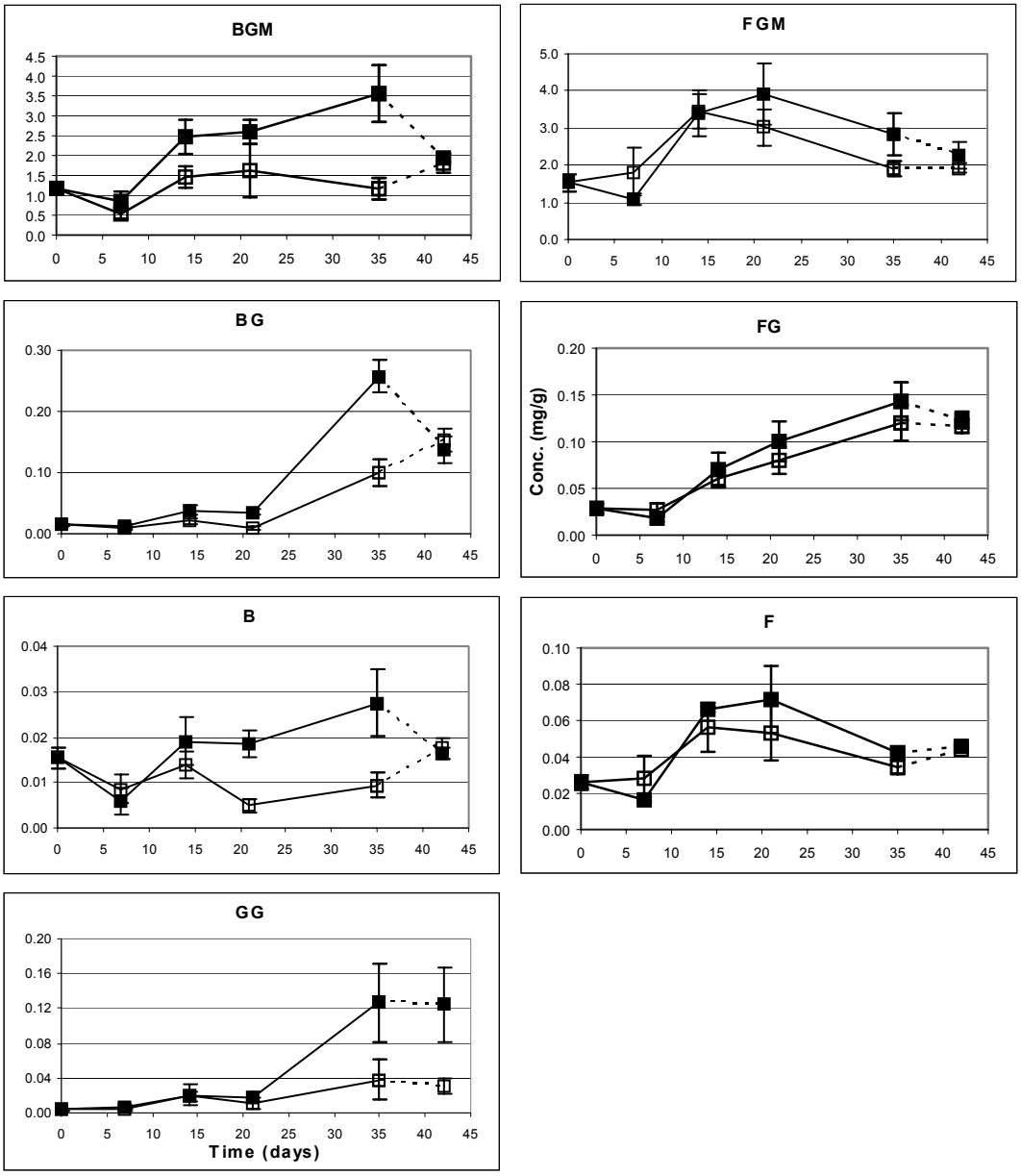


Fig. 2. Effects of waterlogging (□ = normal; ■ = waterlogged) over time on the concentrations (mg/g fresh wt) of seven isoflavones in *T. pratense* leaves in LC–TS-SIM MS, corrected for weight of leaves. Mean values and standard deviations of the mean are given (n=7; 0 days, n=14). Soil water levels were raised at t=1 day and restored to normal at t=35 days.

The concentrations of the seven isoflavone aglycones, glucosides and acylated glucosides during the experiment are displayed in Fig. 2. As expected, at t = 0 days the concentrations of all isoflavones in the N and W plants were the same and therefore at t = 0 days the two groups were pooled together (n = 14). The concentrations of all isoflavones varied in a compound-specific way. For F, FG and FGM, the changes were essentially independent of the water level conditions, with a constant increase in FG during the experimental period. For B, BG, BGM and GG a significant increase of the concentrations

due to waterlogging was observed. BG and GG remained virtually constant up to 21 days, then, between 21 and 35 days they increased 2–3-fold in the W plants due to waterlogging. In contrast, B and BGM differences due to waterlogging occurred already after some 10 days. The data set was evaluated with repeated-measures ANOVA. The results are shown in Table 3 and confirm our findings. The level of significance is displayed for the effects of soil water level, time of exposure and their interaction, *i.e.* does the effect of soil water level change over time.

At t = 35 days, normal soil water conditions were restored for the W plants, and at t = 42 days the concentrations of all isoflavone aglycones and conjugates except GG had returned to values equal to those for the N plants (*cf.* Fig. 2). In all cases, except for FG and BG, these values were equal to the values of t = 0 days, and even for these two analytes, the differences were not large. The results of the independent samples t-test to check whether isoflavone concentrations decreased to the level of the control treatment after restoring the original soil moisture conditions confirm this. There was a significant difference between waterlogged and non-waterlogged plants only for GG, which means that at t = 42 days the effect of waterlogging on the concentration of GG continued. For the other isoflavones and their conjugates, there was no such significant difference at t = 42 days: their concentrations decreased to the level of the control treatment.

Table 2. Level of significance for the effects of soil water level, time of exposure and interaction.

Flavonoid	Soil water level	Time	Interaction
B	**	*	*
BG	**	***	n.s.
BGM	***	***	n.s.
GG	***	*	*
F	n.s.	**	n.s.
FG	n.s.	***	n.s.
FGM	n.s.	***	n.s.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. not significant.

7.4 Discussion

The observed changes in the roots and colour of the leaves between the W and N plants indicate that the W plants suffered from oxygen shortage. Non-flood-resistant plants – such as *T. pratense* – under water stress can avoid the oxygen shortage, either by having their lateral roots close to better oxygenated soil surfaces or by developing superficial adventitious roots in response to flooding. Flood-resistant plants can tolerate the stress because they have morphological and anatomical features, *e.g.* aerenchyma, which allow internal oxygen

transport to satisfy the needs of root respiration and also the oxidation of chemically reduced toxins [21,22].

The concentrations of B, BG, BGM and GG in the *T. pratense* leaves were significantly higher under waterlogging conditions; the other isoflavones did not show any significant changes. (*cf.* Fig. 2). The increase in isoflavone levels in *T. pratense* under waterlogged conditions is comparable with the behaviour of three Australian clover species [16]. Because the processes in the roots such as *Rhizobium* infection and formation of root nodules are disturbed in response to waterlogging, it is likely that the isoflavones that are usually involved in the nodulation process and in the arbuscular mycorrhizal symbiosis were accumulated in the leaves of the plant. Earlier studies with *T. semipilosum* [1] and *T. repens* [23] also show that processes in the roots were affected under waterlogged conditions. It is known that flavonoids are involved in response mechanisms to stress conditions in plants [11,12,15,17,21,24,25] and in biological interactions of clover species with microorganisms. In the symbiosis of clover with arbuscular mycorrhizal fungi, flavonoids can stimulate spore germination and hyphal growth [26,27]. The bacterial nodulation (*nod*) genes are essential for the infection of the host root and the establishment of the nodule. The expression of these genes requires the presence of flavonoids as signal compounds, released from the host plant roots, and the *nodD* regulatory protein [28]. Several authors report the involvement of flavonoids in the induction of the expression of *Rhizobium* nodulation genes from leguminous plants, e.g. *T. pratense* [29], *Galega orientalis* [30] and *Lupinus albus* [19].

Edwards *et al.* [4] studied the effect of nodulation on the isoflavonoid concentration in red clover. Red clover plants that were inoculated with *Rhizobium trifolii* were compared with non-inoculated plants. They found that in the leaves of non-inoculated plants the concentrations of BGM and FGM increased with age and the accumulation of BGM in the leaves was suppressed by nodulation, while FGM remained constant. In their study, a 3-week lag period was observed. These observations are in line with our findings: due to waterlogging the nodulation decreases and this coincides with the accumulation of BGM, BG, B and GG in waterlogged plants, whereas the concentrations of FGM, FG and F remain virtually unaffected. The observed changes of isoflavonoid accumulation are most likely caused by signals from the roots that are indicative of the extent of root nodulation.

After restoration of the normal soil water conditions, oxidation processes are stimulated and processes in the root zone such as mycorrhyza infection and root nodule formation are restored. As Fig. 2 suggests, this regeneration process is much faster than the initial response of the isoflavone aglycones and conjugates, *viz.* about one *vs.* over three weeks. After one week the concentrations of all isoflavones except GG returned to the values found for the N plants. For GG the concentration remained higher: there was no decrease after restoring the normal soil water conditions. An explanation for this may be that GG is

biosynthetically the simplest of the isoflavonoids and therefore a key intermediate in the biosynthesis of more complex isoflavonoids [31]. While the other isoflavones may be used to restore the nodulation processes in the roots, the production of G and GG may remain high for the formation of other isoflavonoids such as coumestans and pterocarpans.

As regards the much faster recovery of the oxygenation of the soil when restored to normal conditions compared with the change due to waterlogging – when the plants are exposed to waterlogged conditions, it takes some time before all the oxygen is removed from the soil [24]. Apparently, after some three weeks the soil is completely anaerobic and processes in the roots and in the rhizosphere are being disturbed. When the soil water level is changed to normal again, the diffusion of oxygen into the soil will be relatively fast and the processes in the roots are restored within one week. A rapid recovery of rhizobial nitrogen fixation was earlier found in soybean [32] and supports our interpretation for *T. pratense* in this study. Also, the activity of rhizosphere microorganisms may strongly increase [33].

7.5 Conclusions

This study shows that the concentrations of the free aglycone biochanin (B), and the conjugates biochanin A–7-*O*-glucoside–malonate (BGM), biochanin A–7-*O*-glucoside (BG), and gensitein–7-*O*-glucoside (GG) in *T. pratense* leaves increased due to disturbed root nodulation in response to waterlogging; other isoflavones did not show any significant differences with the control group growing under normal conditions. The concentrations of BG and GG remained virtually constant up to 21 days, while in the next two weeks they increased 2–3-fold. BGM and B increased somewhat more gradually, *i.e.* approx. 3-fold between 7 and 35 days. Isoflavonoids are involved in plant–microbe communications, *i.e.* the regulation of root nodule activity and the establishment of the mycorrhizal association. Due to waterlogging, the root nodulation was disturbed and as a reaction to this disturbance the isoflavonoids in the leaves increased. After restoring the normal soil water conditions, the concentrations of B, BG and BGM rapidly returned to the values that were found for the normal plants, while the concentration of GG remained high. The reason for this exceptional behaviour may be that GG is a central intermediate in the biosynthesis of more complex isoflavonoids.

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7.6 References

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Samenvatting

Het huidige onderzoek is onderdeel van het project 'Wetlands in de Randstad', waarin onderzoekers vanuit de Faculteiten der Aard- en Levenwetenschappen, Exacte Wetenschappen en Economische Wetenschappen samenwerken, en richt zich op de effecten van vernatting van polder- en wetland-gebieden in de druk bevolkte Randstad. Die vernatting beoogt de ecologische waarde van deze gebieden te herstellen. Het doel van het promotie-onderzoek was om adequate methoden te ontwikkelen voor de analyse van flavonoïden – inclusief hun conjugaten – in complexe matrices, en in wetland-planten in het bijzonder. Deze methoden moeten het uiteindelijk mogelijk maken de wijzigingen in flavonoïd samenstelling door ecologische veranderingen als gevolg van vernatting te bestuderen. De flavonoïden werden geselecteerd omdat ze – in tegenstelling tot bijvoorbeeld lignines en tannines – chemisch goed gedefinieerde stoffen zijn. Bovendien bezitten ze chromofore groepen zodat ze met absorptiespectroscopie in het zichtbare en ultraviolette gebied kunnen worden bestudeerd en vertonen ze bruikbare fragmentatie in massaspectrometrie; ook is bekend dat sommige flavonoïden fluoresceren.

In dit proefschrift worden analytisch chemische methoden ontwikkeld voor flavonoïden in planten van de Leguminosae familie (vlinderbloemigen), die veelvuldig voorkomen in nederlandse wetlands; daarbij wordt meestal gebruik gemaakt van *hyphenated* (gekoppelde) technieken. De studie omvat (i) optimalisatie van extractiemethoden, met nadruk op de stabiliteit van bepaalde flavonoïden; (ii) onderzoek naar UV absorptie, fluorescentie, NMR spectroscopie en (tandem) massa spectrometrie van flavonoïden teneinde optimale detectiecondities te bepalen en, als verkenning van de bruikbaarheid in het kader van de wetlands, (iii) een studie naar het effect van vernatting op de concentraties van de belangrijkste flavonoïden in de bladeren van rode klaver (*T. pratense*) onder vernatte condities. Uiteraard staan deze drie punten niet los van elkaar maar vertonen ze een wederzijdse beïnvloeding.

Na de inleiding in **Hoofdstuk 1**, waarin een overzicht wordt gegeven van de 'state-of-the-art' van de uiteenlopende chemische methoden beschikbaar voor de analyse van flavonoïden in een wijde verscheidenheid aan matrices – en waarin het doel van de studie uiteen wordt gezet – wordt het eerste interessante item in de analyse van flavonoïden, de

ontwikkeling van een adequate extractieprocedure, besproken in **Hoofdstuk 2**. Hierin wordt de analyse van isoflavonen, hun glucosiden en glucoside–malonaten in rode klaver met 'reversed-phase' vloeistof chromatografie (LC) gekoppeld met 'atmospheric pressure chemical ionization' massa spectrometrische (APCI-MS), UV absorptie en fluorescentie detectoren besproken. Methanol extracten kunnen het best worden bewaard bij –20^{o}C om ontleding van flavonoïd–glucosiden en glucoside–malonaten voor ten minste één à twee weken te voorkomen, terwijl een hydrolyse vervolgstap bij voorkeur gedurende twee uur bij 83^{o}C moet worden uitgevoerd, zonder het extract aan te zuren. Onder deze condities worden de glucoside–malonaten, formononetine–7-*O*-β-D-glucoside–6″-*O*-malonaat en biochanine A–7-*O*-β-D-glucoside–6″-*O*-malonaat, kwantitatief omgezet in de corresponderende glucosiden terwijl ontleding van deze laatste verbindingen nog verwaarloosbaar is. Hydrolyse bij 83^{o}C in aanwezigheid van zuur verbreekt de flavonoïde-glucose binding en verschaft zo extra informatie over de flavonoïd verdeling in het extract. In LC-gescheiden fracties zijn de malonaten het meest stabiel als ze worden bewaard bij lage temperatuur na droogdampen. De concentratie van de acht belangrijkste flavonoïden in rode klaver ligt tussen de 0,04 en 5 mg per g blad.

In **Hoofdstuk 3** worden de fluoresentie eigenschappen van negentien flavonoïden besproken. Het aantal verbindingen dat zelf fluorescentie vertoont, is beperkt. Drie isoflavonen, te weten formonetin, ononine en daidzeine bleken te fluoresceren. Zij vertoonden grote Stokes verschuivingen (d.w.z. een groot verschil tussen de excitatie- en emissiemaxima), waarschijnlijk door een verandering van de moleculaire structuur ten gevolge van excitatie, namelijk van niet-planair in de S_0, naar planair in de S_1 toestand. Levensduurmetingen met 'time-correlated single-photon counting' spectroscopie werden gedaan om het mechanisme verder op te helderen. Grote Stokes verschuivingen impliceren een hoge selectiviteit zodat de fluorescerende isoflavonen gemakkelijk gedetecteerd kunnen worden in planten die ook andere fluorescerende stoffen bevatten. Uiteindelijk werd gebruik gemaakt van LC met een methanol–water gradiënt en fluorescentiedetectie met een groot golflengteverschil tussen excitatie en emissie.

In **Hoofdstuk 4** worden de analytische prestaties van vier LC–MS modes (APCI, ESI; positieve ionisatie en negatieve ionisatie) vergeleken voor twee massaspectrometers, een triple-quadrupole en een ion-trap instrument, met vijftien flavonoïden als testverbindingen. Er werden twee organische modifiers, methanol en acetonitril, en twee buffers, ammonium acetaat en ammonium formiaat, gebruikt. Over het algemeen geeft de eluens-combinatie methanol–ammonium formiaat buffer met een pH van 4,0 de hoogste signaalintensiteiten voor de flavonoïden, met APCI(–)-MS op de eerste, en ESI(–)-MS op de tweede plaats. De signaalintensiteiten en de fragmentatie-reacties in MS/MS op de ion-trap en triple-quadrupole

instrumenten verschillen onderling nauwelijks. Onder optimale condities werden full-scan detectiegrenzen van 0,1–30 mg/l bereikt in de APCI(–) mode; er werden responsverschillen tot twee decades gemeten tussen zowel analieten als ionisatiemodes. LC–MS levert karakteristieke fragmentatiepatronen op voor de flavonoïden en geeft informatie over de aard van het aglycon alsmede de aanwezigheid van suikergroepen, malonaatgroepen en methylsubstituenten. In het algemeen worden drie hoofdfragmenten gevormd, $[M–H]^-$ of $[M+H]^+$, $[M–15]^-$ en $[M–2Gly]^-$ of $[M–Gly]^-$. Verder worden retro Diels-Alder fragmenten gevormd door het verbreken van C-ring bindingen. Adductvorming geeft met mierenzuur $[M+45]^-$ ionen en treedt alleen op bij negatieve ionisatie. MS/MS geeft in de meeste gevallen niet veel extra informatie: over het algemeen worden dezelfde fragmenten gevormd als in full-scan MS. De belangrijkste fragmentaties in de MS^n spectra van de ion-trap en de tandem MS spectra van het triple-quadrupole instrument waren over het algemeen dezelfde. MS^n heeft als extra mogelijkheid dat ook precursor → product ion overgangen kunnen worden vast gesteld.

In **Hoofdstuk 5** worden de flavonoïdgehaltes van verschillende planten uit de familie der vlinderbloemigen bepaald. LC op een C_{18}-gebonden silica kolom met een methanol–ammonium formiaat gradiënt werd gebruikt om de belangrijkste flavonoïden in de bladeren van vier plantensoorten te bepalen. Er werden drie detectiemethoden gebruikt, namelijk diode array **(**DAD) UV, fluorescentie en (tandem) MS. LC–DAD UV werd gebruikt voor een algemene screening en classificering van de aanwezige flavonoïden, en voor de berekening van het totale flavonoïd gehalte. LC–FLU werd gebruikt om de aanwezige isoflavonen op basis van hun eigen fluorescentie selectief te bepalen. De meeste structuurinformatie over de aglyconen, alsmede de suiker- en zuurgroepen in de conjugaten werd verkregen met LC–APCI(–)-MS in de full-scan en extracted-ion modes. Wederom leverde MS/MS niet veel extra informatie op; er werden dezelfde fragmenten gevonden als in full-scan MS. In *T. pratense* L. (rode klaver) en *T. repens* L. (witte klaver) waren de hoofdcomponenten flavonoïd–glucoside–(di)malonaten, terwijl *T. dubium* L. (kleine hopklaver) en *L. corniculatus* L. (rolklaver) voornamelijk flavonoïd–(di)glycosiden bleken te bevatten. Zogenaamde satelliet sets in de LC–MS chromatogrammen, afkomstig van een aglycon en het glucoside en/of de glucoside–malonaten of –acetaten ervan, waren in grote mate aanwezig in *T. pratense*, waar dertien van zulke sets werden geïdentificeerd. Geheel afwijkend werd hoogstens één satellietset gevonden in de bladeren van de andere drie planten. Opmerkelijk is het verschil in de belangrijkste aglyconen en suikers voor de vier plantensoorten – bijvoorbeeld formononetine en biochanine A in het geval van *T. pratense*, maar kaempferol in het geval van *L. corniculatus*. Voor wat betreft de suikers is glucose het meest prominent in *T. pratense*, maar glucose en rhamnose bij *T. dubium*. De resultaten voor *T. pratense* bleken vergelijkbaar met literatuurgegevens, terwijl er voor de andere soorten weinig overeenkomst werd gevonden. De totale flavonoïdgehaltes variëerden van 50–65 mg/g blad voor *L.*

corniculatus en *T. dubium*, tot 15 mg/g voor *T. pratense* en slechts 1 mg/g voor *T. repens*. De gedetailleerde structuren van sommige isoflavon–glucoside–malonaat isomeren in *T. pratense* kon in Hoofdstuk 5 nog niet worden opgehelderd. Dit werd gedaan in vervolgonderzoek waarin NMR (Nuclear Megnetic Resonance) te hulp wordt geroepen.

Hoofdstuk 6 illustreert de mogelijkheden van LC–NMR als aanvullende techniek op LC–MS(/MS). Deze methode werd – tezamen met off-line twee-dimensionale NMR – succesvol ingezet voor het verkrijgen van structuurinformatie die het mogelijk maakt om de belangrijkste isoflavon–glucoside–malonaat isomeren in extracten van bladeren van *T. pratense* van elkaar te onderscheiden. Matrix solid-phase dispersion (MSPD) werd gebruikt als monstervoorbewerkingstechniek om voldoende hoge analietconcentraties te verkrijgen voor LC–NMR. Stop-flow reversed-phase LC–proton NMR werd uitgevoerd met een gradiënt van gedeutereerd water en gedeutereerd acetonitril. De posities van de glucosegroep op het flavonoïd aglycon, en van de malonaatgroep op de glucose werden bepaald met behulp van off-line COrrelation SpectroscopY (COSY) en Nuclear Overhauser Enhancement SpectroscopY (NOESY) experimenten. Op basis van de MS/MS fragmentatiepatronen en de NMR spectra konden de twee formononetine–glucoside–malonaat isomeren worden geïdentificeerd als het 7-*O*-β-D-glucoside–6″-*O*-malonaat en het 7-*O*-β-D-glucoside–4″-*O*-malonaat; dwz. ze verschillen alleen maar in de substitutiepositie van de malonaatgroep op de glucosidering. De biochanine A glucoside–malonaat isomeren daarentegen hebben zeer verschillende structuren. De meest voorkomende en laatst eluerende isomeer is biochanine A–7-*O*-β-D-glucoside–6"-*O*-malonaat – de andere isomeer is 5-hydroxy-7-methoxyisoflavon–4′-*O*-β-D-glucoside–4″-*O*-malonaat: de posities van de methoxy groep en de glucoside–6″-*O*-malonaat groep op het flavonoïd skelet blijken te zijn omgewisseld.

In Hoofdstuk 5 werd geconcludeerd dat van de vier bestudeerde plantensoorten *T. pratense* de hoogste flavonoïd concentraties bevat. Deze plant werd daarom uitgekozen om verder te worden bestudeerd onder vernatte condities. In **Hoofdstuk 7** werd de geoptimaliseerde LC–UV–APCI(–)-MS methode gebruikt om de invloed van verstoorde wortelknolletjes-vorming op de samenstelling van isoflavonoïd–glucoside–malonaten, glucosiden en aglyconen in de bladeren vast te stellen. Isoflavonoïden zijn betrokken bij de regulering van wortelknolletjes-activiteit en het aanzetten tot symbiose met mycorrhiza. Er werd gevonden dat, in reactie op een met water verzadigde bodem, de concentraties van het vrije aglycon, biochanine A, en van de conjugaten biochanine A–7-*O*-glucoside–malonaat, biochanine A–7-*O*-glucoside, en genistein–7-*O*-glucoside in de bladeren met een factor twee à drie toenamen na een periode van drie weken. De concentraties van de andere onderzochte isoflavonen – het aglycon formononetine, en de conjugaten formononetine–7-*O*-glucoside–malonaat en formononetine–7-*O*-glucoside – vertoonden daarentegen geen

significante veranderingen als gevolg van het verhoogde waterpeil. Na herstel van de normale grondwatercondities namen de concentraties van biochanine A en zijn twee conjugaten weer snel af tot hun beginwaarden, terwijl de concentratie van genistein–7-*O*-glucoside hoog bleef. Dit doet vermoeden dat de laatstgenoemde verbinding geschikt kan zijn als een chemische indicator om vernattingsinvloeden te bestuderen. Verder ecologisch onderzoek is nodig om deze suggestie te bevestigen.

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List of Publications

This thesis is based on the following papers, which have been or will be published as regular contributions to scientific journals:

- E. de Rijke, A. Zafra-Gómez, F. Ariese, U.A.Th. Brinkman, C. Gooijer, Determination of isoflavone–glucoside–malonates in *Trifolium pratense* L. (red clover) extracts: Quantification and stability studies. *J. Chromatogr. A* 931 (2001) 55-64 (Chapter 2).

- E. de Rijke, H. Joshi, H.R. Sanderse, F. Ariese, U.A.Th. Brinkman, C. Gooijer, Natively fluorescent isoflavones exhibiting anomalous Stokes' shifts. *Anal. Chim. Acta* 468 (2002) 3-11 (Chapter 3).

- E. de Rijke, H. Zappey, F. Ariese, C. Gooijer, U.A.Th. Brinkman, Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. *J. Chromatogr. A* 984 (2003) 45-58 (Chapter 4).

- E. de Rijke, H. Zappey, F. Ariese, C. Gooijer, U.A.Th. Brinkman, Flavonoids in Leguminosae: analysis of extracts of *T. pratense* L., *T. dubium* L., *T. repens* L. and *L. corniculatus* L. leaves using liquid chromatography with UV, mass spectrometric and fluorescence detection. *Anal. Bioanal. Chem.* 378 (2004) 995-1006 (Chapter 5).

- E. de Rijke, F. de Kanter, F. Ariese, U.A.Th. Brinkman and C. Gooijer, Liquid chromatography coupled to nuclear magnetic resonance spectroscopy for the identification of isoflavone–glucoside–malonates in *T. pratense* L. leaves. *J. Sep. Sci.* 27 (2004) 1061-1070 (Chapter 6).

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Additional paper:

- J. Kuijt, E. de Rijke, U.A.Th. Brinkman, C. Gooijer, Practical implementation of quenched phosphorescence detection in capillary electrophoresis, *Anal. Chim. Acta* 417 (2000) 15-17.